ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 361

THE CHEMICAL CONTROL OF VENTILATION

BY

SØREN CLAUS SØRENSEN

STOCKHOLM 1971

This review is based on the following previous publications:

I SORENSEN, S. C. and J W SEVERINGHAUS. Respiratory sensitivity to acute hypoxia I ma born at sea level living at high altitude J appl. Physiol. 1968a. 25 211 216.

II SORENSEN, S. C. and J W SEVERINGHAUS. Irreversible respiratory insensitivity to acute hypoxia in man born at high ltitude. J appl. Physiol. 1968b 25 217 220.

III SORENSEN S. C. and J W SEVERINGHAUS. Respiratory insensitivity to acute hypoxia per sisting after correction of tetralogy of Fallot. J appl. Physiol. 1968c. .5 221 223.

IV SORENSEN, S. C. and J C. CRUZ. Ventilatory response to single breath of CO in O_2 in normal man at sea level and high altitude. J appl. Physiol. 1969 27 186–190.

V HORNBEIN, T F and S. C. SORENSEN. Ventilatory response to hypoxia and hypercapnia in cats living at high altitude. J appl. Physiol. 1969 27 834–836.

VI MINES, A. H nd S. C. SORENSEN. Ventilatory response to acute hypoxia in goat kids born at high altitude. Acta physiol. Scand. 1969 77 439–441

VII MINES, A. H and S. C. SORENSEN. Ventilatory responses of w ke normal goats d ri g acute and chronic hypoxia. J appl. Physiol. 1970. 28. 826–831

VIII SORENSEN, S. C. and A. H MINES. Ventilatory responses to acut and chronic hypoxia in goats after sinus nerve section J ppl. Physiol. 1970. 28 83 -835.

IX SORENSEN, S. C. Ventilatory cclimatization to hypoxia i rabbits after denervation of peripheral chemoreceptors. J appl. Physiol. 1970. 28 836–839

X SORENSEN S. C. and J W SEVE INGHAUS. Effect of cerebral acidosis on the CSF blood potential difference. Am. J Physiol. 1970. 219 68–71

XI MINES, A. H and S. C. SORENSEN. Cha ges in the electrochemical potential difference for HCO between blood and csf and in csf lactat concentration during isocarbic hypoxia. Acta. physiol. Scand. 1971 81 225–233

XII MINES, A. H C. C. MORRILL, and S. C. SORENSEN. The effect of isocarbic metabolic cidosis m blood on [H] and [HCO_3^-] i csf with deductions bout the regulation of active transport of H /HCO between blood and csf Acta physiol. Scand. 1971 81 234–245

Forsvaret finder sted torsdag den 1. april 1971 kl. 14 præcis i
annexauditorium A, Studiestræde 6, o. g.

ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 361

From Cardiovascular Research Institute and Department of Physiology
University of California,
San Francisco Medical Center San Francisco California 941-2, U. S. A.
and Institute of Medical Physiology University of Copenhagen,
Juliane Mariesvej 30, Copenhagen, Denmark

THE CHEMICAL CONTROL OF VENTILATION

BY

SØREN CLAUS SØRENSEN

STOCKHOLM 1971

Denne afhandling

er i forbindelse med omstående tidligere publicerede afhandlinger af det

lægevidenskabelige fakultet ved Københavns universitet

antaget til offentlig at forsvares for den medicinske doktorgrad

København, den 7. januar 1971

FRANK LUNDQUIST
h. a. dec.

Printed in Denmark

Andelsbogtrykkeriet i Odense

This review is based on the following previous publications

I Sørensen, S. C. and J. W. Severinghaus. Respiratory sensitivity to acute hypoxia in man born at sea level living at high altitude. J. appl. Physiol. 1968a. 25. 211–216.

II Sørensen, S. C. and J. W. Severinghaus. Irreversible respiratory insensitivity to acute hypoxia in man born at high altitude. J. appl. Physiol. 1968b. 25. 217–220.

III Sørensen, S. C. and J. W. Severinghaus. Respiratory insensitivity to acute hypoxia persisting after correction of tetralogy of Fallot. J. appl. Physiol. 1968c. 25. 221–223.

IV Sørensen, S. C. and J. C. Cruz. Ventilatory response to a single breath of CO_2 in O_2 in normal man at sea level and high altitude. J. appl. Physiol. 1969. 27. 186–190.

V Hornbein, T. F. and S. C. Sørensen. Ventilatory response to hypoxia and hypercapnia in cats living at high altitude. J. appl. Physiol. 1969. 27. 834–836.

VI Mines, A. H. and S. C. Sørensen. Ventilatory response to acute hypoxia in goat kids born at high altitude. Acta physiol. Scand. 1969. 77. 439–441.

VII Mines, A. H. and S. C. Sørensen. Ventilatory responses of unanesthetized normal goats during acute and chronic hypoxia. J. appl. Physiol. 1970. 28. 826–831.

VIII Sørensen, S. C. and A. H. Mines. Ventilatory responses to acute and chronic hypoxia in goats after sinus nerve section. J. appl. Physiol. 1970. 28. 832–835.

IX Sørensen, S. C. Ventilatory acclimatization to hypoxia in rabbits after denervation of peripheral chemoreceptors. J. appl. Physiol. 1970. 28. 836–839.

X Sørensen, S. C. and J. W. Severinghaus. Effect of cerebral acidosis on the CSF-blood potential difference. Am. J. Physiol. 1970. 219. 68–71.

XI Mines, A. H. and S. C. Sørensen. Changes in the electrochemical potential difference for HCO_3^- between blood and csf and in csf lactate concentration during isocarbic hypoxia. Acta. physiol. Scand. 1971. 81. 225–233.

XII Mines, A. H., C. C. Morrill, and S. C. Sørensen. The effect of isocarbic metabolic acidosis in blood on $[H^+]$ and $[HCO_3^-]$ in csf with deductions about the regulation of active transport of H^+/HCO_3^- between blood and csf. Acta physiol. Scand. 1971. 81. 234–245.

Contents

Chapter 7

Acknowledgements

The experimental work which forms the basis of this review was performed while I was an International Postdoctoral Research Fellow and an Assistant Research Physiologist at the Cardiovascular Research Institute, University of California, San Francisco Medical Center from 1966 to 1969 I am greatly indebted to Dr Julius H Comroe, Jr who created and directs this institute, for the opportunity to work in such a stimulating environment.

A great deal of the stimulation came from Dr John W Severinghaus with whom part of the experimental work was performed I benefitted immensely from our frequent discussions, flavoured by his enthusiasm, inquisitiveness and unrestrained imagination

I am also indebted to Dr Ralph H Kellog, who generously provided me with research facilities in his laboratory for more than two years.

A large part of the experimental work was performed at the University of California High Altitude Research Station at White Mountain, California and at Instituto de Investigaciones de la Altura, Universidad Peruana Cayetano Heredia, Peru The projects have depended upon the collaboration of so many people that I cannot name each one here, but I would reexpress my gratitude to them all now

I also want to thank Dr Poul W Kruhoffer Institute of Medical Physiology University of Copenhagen for many valuable discussions.

The research was supported in part by the following grants from the National Institutes of Health, Bethesda, Maryland U.S.A. Program project grants HE-06285 and HE-08866, Research grant GM-09262, International Postdoctoral Research Fellowship 1-F05-TW-1062, Training grant 1-T1-GM-00927 Research Career Award 5-K6-HE-19412, Research Career Development Award 5-K3-HE-9617 and in part by a research stipend (kandidatstipendium) from University of Copenhagen and by grants from "Kobmand i Odense Johann og Hanne Weimann f Seedorf's legat"

Introduction

In most mammals the primary function of ventilation of the airways is to maintain an adequate gas exchange during varying metabolic demands. In many mammals however ventilation is also essential in heat exchange (panting) and ventilation may be transiently subjugated to other functions such as coughing, sighing, yawning and talking. Maintenance of these functions requires input from a variety of receptors and a center to integrate the different functions. The complexity of the systems is illustrated by the panting animal where rapid shallow breathing for the purpose of heat exchange might alternate with tidal breathing for the purpose of gas exchange. We shall in this review deal with the mechanisms by which an adequate gas exchange is achieved. We must however be continually aware of the fact that the information obtained with an animal standing still in a stanchion or the human subject comfortably reclined on a couch might be of only limited relevance to the much more complex situations during either exercise or heat stress.

The purpose of gas exchange in the lungs is to deliver oxygen to the tissues and to remove the produced CO_2 The major role of oxygen demand in regulating the level of ventilation is illustrated by the constancy of arterial Po_2 during the phylogenetic transition from water breathers to air breathers in contrast to a twenty-fold increase in Pco_2 (Fig. 1) From this trend one could expect that ventilation was regulated mainly by sensory mechanisms responding to changes in Po_2. The increase in Pco_2 during phylogenesis is however compensated by mechanisms which have maintained acid-base constancy despite changes in Pco_2 (Rahn 1966) and in air breathers, at least, ventilation changes in response to small changes in arterial Pco_2

In mammals two sensory systems with different characteristics have been demonstrated 1) The peripheral chemoreceptors, the carotid and aortic bodies and 2) central chemosensitive areas.

The peripheral chemoreceptors were described anatomically by De Castro (1926) and their functional significance was described by Heymans and Heymans (1927) who showed that ventilation was stimulated by perfusing through the aortic arch with hypoxic blood from another animal. The role of the peripheral chemoreceptors in control of ventilation is considered mainly to be related to their sensitivity to changes in arterial Po_2 (Comroe 1964) although they also respond to changes in arterial Pco_2 and pH (Hornbein, Griffo and Roos 1961 Hornbein and Roos 1963 Gray 1968). The central chemosensitive areas are located in the brainstem and are sensitive to pH changes in their environment (Leusen 1954a,b, Mitchell et al 1963 Pappenheimer et al. 1965) Because they respond slowly to metabolic acid-base changes in the blood they function mainly as Pco_2 sensors.

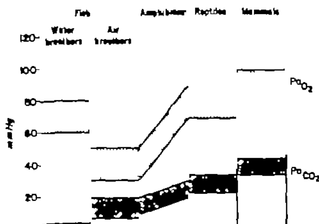


Fig. 1 Diagrammatic representation of changes in blood gas values during the transition from water breathing to air breathing in vertebrates (Redrawn from Lenfant, Johansen and Hanson 1970)

In the following we shall first describe the peripheral chemoreceptors and the central chemosensitive areas. We shall then examine how the two systems interact during acute changes in arterial P_{O_2}, P_{CO_2} and pH We shall then review and discuss evidence that man who is born and has been living in a hypoxic environment during early childhood shows an irreversibly blunted peripheral chemoreceptor response to hypoxia. On this basis we shall evaluate the concept that the ventilatory changes observed during chronic hypoxia are secondary to the reflex response to hypoxia mediate by the peripheral chemoreceptors. We shall review evidence that ventilation deed increases during chronic hypoxia in man with a blunted peripheral chemore- response to hypoxia and in animals surgically deprived of their peripheral chemoreceptors. We shall discuss the different mechanisms which might be responsible for the ventilatory changes during chronic hypoxia and on this background we shall discuss the mechanisms which might be responsible for the ventilatory adjustments during chroni acid-base disturbances.

Several reviews and symposia have in recent years dealt with various aspects of the chemical control of ventilation (Comroe 1964 Kellogg 1964 Cunningham and Lloyd, ed. 1963 Brooks, Kao and Lloyd ed 1965 Torrance, ed 1968). These all provide excellent entries to the large literature about the chemical control of ventilation. I have therefore in this review been very selective in terms of references A selection of references is by nature subjective and someone may accuse me of chauvinism although I have attempted to give justice to differing viewpoints.

Chapter 1

The Peripheral Chemoreceptors

The peripheral chemoreceptors respond to changes in arterial Po_2, Pco_2 and pH. They are the carotid and aortic bodies. Much of the information concerning their function has been obtained from studies of the carotid bodies, which can be isolated, perfused, and their innervation in mammals is readily accessible for recording with electrodes. The aortic bodies are fairly inaccessible and much of the evidence for their specific function is indirect. Therefore many generalisations about the function of the peripheral chemoreceptors as an entity is based on evidence from the carotid bodies only

Nearly all direct studies of carotid chemoreceptor function have been performed in cats, which may limit the validity of generalisations with regard to other species. We have for instance found a species difference in the development of a blunted peripheral response to hypoxia during chronic hypoxia in childhood (see Chapter 4). Also the pronounced differences in morphology of the carotid bodies between species (see de Koch 1959) may indicate differences in their functional characteristics.

The carotid bodies The carotid bodies are tiny structures (diameter 1 to 2 mm in the cat, 5 mm in adult man) situated bilaterally in the carotid bifurcation. They are highly vascularized, which led De Castro (1926) to suggest, that they might sense the chemical composition of the blood. Heymans and Heymans (1927) showed that they indeed possess this function by demonstrating that ventilation was stimulated when an animal was perfused through the aortic arch with blood an animal breathing an air mixture low in oxygen A few years later they delineated the location and function of the carotid bodies and demonstrated that they were stimulated both by hypoxia and hypercarbia (Heymans, Bouckaert and Dautrebande 1930)

The carotid body is supplied by two nerves. One of these is the sinus nerve, which contains the chemoreceptor afferent fibers (Heymans and Neil 1958 Comroe 1964). The other nerve is a postganglionic branch of the sympathetic superior cervical ganglion (Gerard and Billingsley 1923).

The sinus nerve has previously been assumed to carry only afferent fibers from the carotid body and the baroreceptors but recently efferent impulse traffic has been recorded in the sinus nerve (Biscoe and Sampson 1967). Two types of potentials have been recorded from the central part of the cut sinusnerve. The one type is generated by sympathetic fibers and they disappear when the cervical sympathetic trunk is sectioned. The second type of potentials is affected by changes in systemic Po_2 and Pco_2. A lowering of Po_2 and an increase in Pco_2 increases the rate of firing in these fibers even when the vagus nerves, the cervical sympathetic trunks and the contralateral sinus nerve have been cut. It has however not been possible to demonstrate any

effect of stimulation of efferent fibers on the impulse traffic in afferent fibers, and the function of the former remain unknown (Biscoe personal communication)

The functional significance of the sympathetic nerve supply to the carotid body is still uncertain. Many sympathetic fibers probably bypasses the carotid body but others innervate the vasculature (Daly Lambertsen and Schweitzer 1954). An increased sympathetic tone might increase the afferent impulse traffic from the carotid and aortic bodies and increase ventilation through an effect on blood flow to the carotid bodies (Floyd and Neil 1952, Eyzaguirre and Lewin 1961 b, Cunningham et al. 1963 Lee, Mayou and Torrance 1964 Biscoe and Purves 1967). The implications with regard to carotid body function during physiological events in the intact organism remain unsolved.

The carotid body is stimulated by a lowering of P_{O_2} in blood perfusing the carotid body Afferent activity can be recorded from the whole nerve at a P_{O_2} of 100 mm Hg, and when P_{O_2} is lowered the nerve activity increases (Hornbein, Griffo and Roos 1961 Eyzaguirre and Lewin 1961 a, Biscoe, Sampson and Purves 1967) Recordings from few fiber preparations suggest that the activity recorded from the whole nerve when lowering P_{O_2} results both from increased activity in individual fibers and from recruitment of new fibers with different response characteristics (Biscoe, Sampson and Purves 1967)

The afferent activity at any given P_{O_2} increases with an increase in arterial P_{CO_2} or a lowering of arterial pH The effect of changes in arterial P_{O_2} and P_{CO_2} or pH on the carotid body are not simple additive because the effect of a combined lowering of P_{O_2} and an increase in P_{aCO_2} is greater than the sum of the effects of each individual alterations (Hornbein, Griffo and Roos 1961) This effect is illustrated in fig. 2. The multiplicative interaction between P_{O_2} and P_{CO_2} or pH has been termed "positive interaction" by respiratory physiologists.

Several studies have examined whether stimulation of the carotid body by a change in P_{CO_2} can be quantitatively described as a single function of the resulting change in arterial pH or whether there is a "CO_2 effect" Hornbein and Roos (1963) concluded that the afferent impulse traffic in the sinus nerve is a single function of arterial pH during both metabolic and respiratory changes in arterial pH This conclusion disagreed with earlier findings (Joels and Neil 1960) and it has been contended again recently (Biscoe, Sampson and Purves 1967) although the concept that the stimulation caused by an increase in P_{CO_2} is due to a pH change somewhere in the carotid body has not been questioned.

In a very elegant study Gray (1968) found that the response to CO_2 was a response to pH in a compartment in close contact with blood By studying the isolated perfused carotid body he could demonstrate a temporal dispersion of the responses to P_{CO_2} changes and to "metabolic" pH changes of the perfusion fluid The response to a change in P_{CO_2} was faster but the time difference was only a few seconds. He concluded that the carotid bodies respond to changes in their extracellular pH and they are capable of responding to even the most acute metabolic acid-base disturbances without significant delay

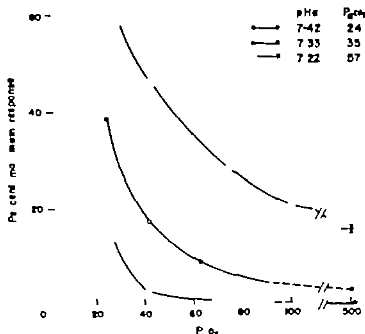


Fig. 2. Averaged neural discharge from the carotid body f cat as a function of arterial Po_2 at three levels of arterial Pco_2–pH achieved by changi g ventilation (From Hornbein 1968).

The temporal dispersion of the responses to acute metabolic and respiratory changes in blood pH provides an explanation for the apparent adaptation of the carotid body response to an acute change in Pco_2 (Black, McCloskey and Torrance 1966). Following hypercapnic acidification the chemoreceptor response overshoots before it reaches a plateau after some 10 to 20 seconds. This may represent the time required for HCO_3^- equlibration blood and the less buffered extravascular fluid following an acute and simultaneous increment in Pco_2 in the two compartments.

Blood flow and metabolism The relation between blood flow to and metabolism of the carotid body has been of interest in attempts to understand the function of the carotid body The question is Why does the carotid body sense "hypoxia" when the arterial Po_2 is lowered from for instance 90 to 70 mm Hg? If the blood flow was low compared to the rate of O_2-consumption the $(a-v)_{O_2}$-difference would be large and tissue Po_2 would be low even at high arterial Po_2 s. However all available evidence indicate that the $(a-v)_{O_2}$-difference is minimal and that the tissue Po_2 approaches arterial Po_2. Daly Lambertsen and Schweitzer (1954) found a fairly large O_2-consumption (9 ml/100 g × min) but they also found a very large blood flow (roughly 2000 ml/100 g × min) Recent evidence indicates that the O_2-consumption is as low as that of nervous tissue in general (about 3 ml/100 g × min) (Fay 1970)

The blood flow value found by Daly Lambertsen and Schweitzer (1954) does not give any indication about the $(a-v)_{O_2}$-difference across the capillaries or sinusoids of the glomera because there may be arterio-venous shunts in the carotid bodies (Hey

mans and Neil 1958) However other indirect evidence supports the suggestion that the mean capillary Po_2 *in the carotid body is close to arterial* Po_2 *For example the absence of a carotid body response to "anemic hypoxia" certainly suggests a very* small $(a-v)_{O_2}$-difference. Duke, Green and Neil (1952) found no increase in chemoreceptor discharge when they saturated 70 to 80 / of blood hemoglobin with carbon monoxide although when an even larger fraction of hemoglobin is saturated with carbon monoxide the chemoreceptor discharge increases at a constant arterial Po_2 (Hornbein 1968 Mills and Edwards 1968)

Calculations of "mean" tissue Po_2 from $(a-v)_{O_2}$-*differences require information* about geometry diffusion distances and diffusion coefficients in the chemoreceptor tissue. Torrance (1968) calculated a 10 to 20 mm Hg fall in Po_2 from blood to the chemosensitive cells but he also cautioned about the validity of his assumptions about diffusion characteristics in the carotid body His calculation however suggest that the carotid body which responds to a fall in Po_2 below 100 mm Hg shows some change in *metabolism when the tissue falls below 80 to 90 mm Hg. There has been much spe*culation but little experimental evidence about the metabolic links between a lowered Po_2 and an increased affesent impulse activity The different suggestions have been detailed in a recent publication (Torrance, ed. 1968)

Recent electronmicroscopic studies of the carotid body suggests that the carotid body may not be a simple sensory organ because the glomus cells or type I cells are *innervated by efferent fibers (Biscoe and Stehbens 1966, Biscoe and Stehbens 1967).* This information was substantiated by demonstration of efferent impulse traffic in the sinus nerve as mentioned previously but the role of the efferent innervation in the stimulation of the carotid body by hypoxia is unknown.

The aortic bodies The aortic bodies are fairly inaccessible thus, because it has not been possible to isolate or control their circulation, direct studies have been difficult. Their existence and general location was established by Heymans and Heymans (1927), but the exact localisation and function of the aortic bodies was described by Comroe (1939). The relative contribution of carotid and aortic bodies to the hyperpnea during acute hypoxia may vary between species (Comroe 1964)

Central Chemosensitive Areas

After total denervation of the carotid and aortic bodies ventilation is still stimulated by CO_2 (Gemmill and Reeves 1933 Tenney and Brooks 1966). This demonstrates the presence of chemoreceptors anatomically separate from the carotid and aortic bodies. A greater ventilatory response to hypercapnia than to metabolic acidosis for equivalent changes in arterial pH (Laquer and Verzar 1911 Katsaros et al. 1960, Lambertsen et al 1961) indicate 1) that these receptors respond differently from the carotid body (Hornbein and Roos 1963 Gray 1968) and/or 2) that the receptors are separated from the blood stream by a diffusion barrier permeable to CO_2 but less permeable to H^+ and HCO_3^- Recent studies have provided ample evidence that they are situated in the brain.

The major step towards description of intracranial chemoreceptors was made by Leusen (1954a,b) who demonstrated that perfusion of the ventriculocisternal system with an acid fluid stimulated ventilation while perfusion with an alkaline fluid depressed ventilation. Leusens fundamental observations were confirmed and extended by observations in several laboratories (Loeschcke, Katsaros and Lerche 1958, Mitchell et al. 1963 Pappenheimer et al. 1965).

Mitchell et al. (1963) suggested that changes in composition of cerebrospinal fluid (csf) exert their effect on ventilation via specific "chemoreceptors" which are sensitive to changes in pH in their environment. They delineated two areas on the surface of medulla from which they could elicit ventilatory changes when they changed pH in the bathing fluid. They suggested that these areas contain the central chemoreceptors per se because they could induce apnea by applying local anesthesia to these areas in anesthetized cats. However apnea is not induced when applying local anesthesia to these areas in the unanesthetized decerebrate cat (Cozine and Ngai 1967) and subsequently other superficial pH sensitive areas in the brainstem were delineated (Schläfke, See and Loeschcke 1970)

There has been no morphological evidence for the existence of receptor cells in the delineated areas hence these areas of high sensitivity may merely reflect that pH sensitive respiratory center neurons are located superficially in these areas in the brain stem. Pappenheimer et al (1965) attempted to measure the sensitivity of central chemosensitive areas by changing pH in fluid perfused from the lateral ventricle to cisterna magna in the awake goat. Lowering pH by changing [HCO_3^-] in the perfusion fluid was less than half as effective as a ventilatory stimulus than the same pH change caused by a change in inspired P_{CO_2} They concluded that the central chemosensitive areas are located at some distance from bulk csf Assuming that the stimulation of ventilation by a change in inspired P_{CO_2} and by a change in [HCO_3^-] in ventriculo-

cisternal perfusion fluid is mediated through a single population of chemosensitive cells, they placed the chemosensitive areas along the gradient for [HCO_3^-] between csf and blood. Their results may however also be interpreted as indicating that CO_2 stimulates ventilation through some sites which do not reach equilibrium with [HCO_3^-] in ventriculo-cisternal perfusion fluid. These other sites may be located in the brain or may be the peripheral chemoreceptors, which were disregarded by Pappenheimer et al.

We must conclude that there are areas in the brain stem through which ventilation can be stimulated by a lowering of pH in the extracellular fluid. There might be several areas and there might be specific receptors but it might also be an effect of pH on *certain respiratory neurons. It has not been resolved whether the pH effect is due* only to the changes in extracellular fluid pH or whether it is due to changes in intra cellular pH To resolve this problem will require a definite anatomical localisation *of the chemosensitive areas, and an analysis of the temporal dispersion of the re-*sponses to "metabolic and respiratory" changes in their environment, similar to the analysis performed for the carotid body (Gray 1968)

The sensitivity of the central chemosensitive areas may be expressed in terms of the ventilatory response to a pH change in brain extracellular fluid. Because the ventilatory responses are different when csf pH is changed by changing [HCO_3^-] in ventriculo-cisternal perfusion fluid and when it is changed by changing inspired P_{CO_2} only a minimum and a maximum value for the sensitivity can be calculated from data obtained in the awake goat by Pappenheimer et al. (1965). The minimum value is the ventilatory response to pH changes caused by changing [HCO_3^-] in ventriculo-cisternal perfusion fluid but the minimum value reflects the sensitivity of the central chemosensitive areas *only if [H^+] at the receptor sites is in equilibrium with [H^+] in ventriculocisternal perfu*sion fluid as suggested by Mitchell et al. (1963) The maximum value is the ventilatory response to pH changes incurred by changing inspired P_{CO_2}. This value reflects the sensitivity of the central chemosensitive areas if the ventilatory response to an increase in inspired P_{CO_2} is mediated only through central chemosensitive areas, which may not all reach equilibrium with [H^+] in ventriculo-cisternal perfusion fluid.

When pH is decreased 0 1 unit in ventriculo-cisternal perfusion fluid by changing the [HCO_3^-] ventilation increases from 5 L/min to 10 L/min The same increase in ventilation is associated with only a 0.05 pH unit *fall* in csf pH when CO_2 is inhaled. The sensitivity of all chemosensitive areas in the brain must therefore lie between these two values if they respond to changes in brain extracellular fluid pH the remainder of the response to *inhaled* CO_2 *being due to stimulation of the peripheral* chemoreceptors.

Severinghaus et al. (1963) computed the ventilatory response to csf pH changes *in man during* CO_2 *inhalation assuming a minimal contribution of the peripheral* chemoreceptors. The ventilatory response to CO_2 was variable among the four subjects which they examined, but the computed Δ ventilation/Δcsf pH was in general somewhat larger than that found in goats.

Chapter 3

Acute Ventilatory Responses

A lowering of arterial Po_2 or a metabolic change in blood acid base composition will affect ventilation within seconds through the peripheral chemoreceptors. The resulting change in Pco_2 will modify the stimulus to the central chemosensitive areas before a new steady state is attained within some minutes. These changes in ventilation may be called the acute ventilatory responses. If a low Po_2 or a blood acid-base change is maintained for hours or days ventilation will change further approaching a new steady state within days. The mechanisms which result in the chronic ventilatory adjustments are initiated immediately upon a change in blood composition but during the first minutes after a change in blood composition these latter mechanisms do not affect ventilation significantly and we can ignore them when measuring the acute ventilatory response.

Hypoxia When Po_2 in inspired air is decreased ventilation in most cases increases within seconds due to stimulation of the peripheral chemoreceptors. When an animal is deprived of the peripheral chemoreceptors ventilation during acute exposures to hypoxia, is no longer stimulated but is often depressed (Gemmill and Reeves 1933 Watt, Dumke and Comroe 1943 Mitchell 1965 Sorensen and Mines 1970) indicating that the ventilatory stimulation from acute hypoxia is mediated through the peripheral chemoreceptors.

However when the peripheral chemoreceptors are intact the ventilatory response to acute hypoxia is also determined by the sensitivity to CO_2 of the peripheral chemoreceptors and the central chemosensitive areas. When arterial Po_2 is lowered the peripheral chemoreceptor drive to ventilation increases but the ensuing hypocapnia due to hyperventilation decreases both the peripheral and the central chemical drive to ventilation (Gökhan and Winterstein 1958 Leusen and Demester 1960) and the eventual increase in ventilation is much less than what would result from the increase in peripheral chemoreceptor drive alone. The changes in peripheral and central drive to ventilation and in ventilation is illustrated schematically in fig. 3

The extent to which hypocapnia alters the ventilatory response to hypoxia depends on the sensitivity of the peripheral chemoreceptors and the central chemosensitive areas to changes in Pco_2 (or rather pH) In fig. 4 the increases in ventilation during acute hypoxia are compared when arterial Pco_2 is allowed to fall and when it is kept constant (iso-capnia) by adding CO_2 to the inspired air

For measurements of the ventilatory response to hypoxia per se methods which are based on iso-capnia rather than a falling Pco_2 are more valid. Iso-capnia can be accomplished by adding CO_2 to the inspired air while the inspired Po_2 is lowered (Loeschcke and Gertz 1958) CO_2 analyzers which can measure airway Pco_2 contin-

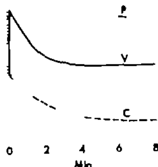


Fig. 3 Diagrammatic illustration of the acut changes in peripheral (P) and central (C) chemical drive to ventilation and in ventilation ($\dot{V}$) when Po_2 in inspired air is lowered. The diagram is not a true quantitative description of the relative changes because the relative contribution from peripheral and central chemosensitive areas is largely unknown. The same warning applies to Fig. 6, 8 and 16.

ously have greatly facilitated the maintenance of a constant Pco_2 during exposures to hypoxia.

The "iso-capnic" ventilatory response to hypoxia can also be derived from measurements of the steady state ventilatory response CO_2 (CO_2 response curves) during normoxia and during hypoxia (Fig. 5) If alveolar or arterial Po_2 is kept constant while measuring the ventilatory response to CO_2 the hypoxic CO_2 response curve will during hypoxia be shifted towards a lower Pco_2 and the slope will be increased com-

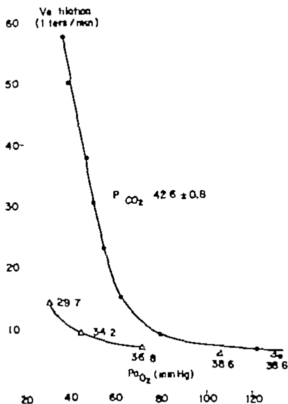


Fig. 4 Ventilatory response to hypoxia in one human subject. The upper curve shows the ventilatory response when alveolar Pco_2 was kept constant at 42 6 mm Hg by adding CO_2 to the inspired gas mixt re. The lower curve shows the ventilatory response when Pco_2 was allowed to fall with hyper ventilation. The numbers beside the lower curve refer to the measured lveolar Pco_2 t each point on the curve (Redrawn from Loeschcke and Gertz 1958).

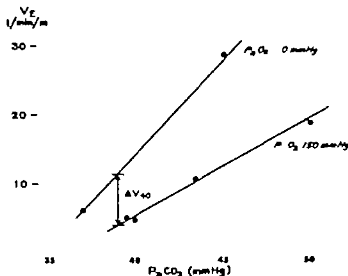


Fig. 5 CO_2 response curves btained in one human subject when the rterial Po_2 was kept consta t at 40 mm Hg and at 150 mm Hg respectively A stand d Pco_2 is obtained by extrapolating the high oxygen CO_2 response curve downward to a ventilation of 4 L/min × m^2 The iso-capnic" ventilatory response to hypoxia (ΔV_{40}) is measured as the change in ventilation when decreasi g arterial Po_2 from 150 mm Hg to 40 mm Hg at standard" Pco_2 (From Sorensen and Severinghaus 1968).

pared with the hyperoxic CO_2 response curve (Nielsen and Smith 1952, Lloyd Jukes and Cunningham 1958). The increase in slope of the CO_2 response curves during hypoxia indicates that the increase in ventilation for a certain increase in Pco_2 is greater when the Po_2 is lower and that the increase in ventilation for a certain decrease in Po_2 is greater when the Pco_2 is higher ("positive interaction ") Consequently the "iso-capnic" increase in ventilation during acute hypoxia depends on the Pco_2 at which it is measured. Therefore when the iso-capnic ventilatory responses to acute hypoxia are compared between different subjects it is necessary to define not only Po_2 but also Pco_2 The "iso-capnic" ventilatory response to hypoxia was measured at the Pco_2 of the resting subject breathing ambient air by Severinghaus, Bainton and Carcelen (1966) and at a Pco_2 which was derived from the hyperoxic CO_2 response curves by Sorensen and Severinghaus (1968 a, b c) (see fig. 5)

Lloyd, Jukes and Cunningham (1958) used a family of CO_2 response curves measured at different alveolar Po_2 s and they performed a mathematical reduction which assumes a common zero-ventilation intercept for all CO_2 response curves in a given individual. Essentially the effect of acute hypoxia on ventilation was expressed as the effect of hypoxia on the slope of the CO_2 response curve The major problem in this approach is the validity of the concept of a common intercept for the CO_2 response curves. The concept has not been validated in most studies where this approach has been applied because the different CO_2 response curves have been drawn on the basis of one or two points assuming a common intercept (Cunningham et al. 1961 Milledge

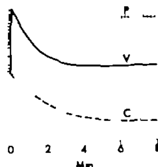


Fig. 3 Diagrammatic illustration of the acute changes in peripheral (P) and central (C) chemical drive to ventilation and in ventilation ($\dot{V}$) when P_{O_2} in inspired air is lowered. The diagram is not a true quantitative description of the relative changes because the relative contribution from peripheral and central chemosensitive areas is largely unknown. The same warning applies to Fig. 6, 8, and 16.

ously have greatly facilitated the maintenance of a constant P_{CO_2} during exposures to hypoxia.

The "iso-capnic" ventilatory response to hypoxia can also be derived from measurements of the *steady state ventilatory response CO_2* (CO_2 response curves) *during* normoxia and during hypoxia (Fig. 5) If alveolar or arterial P_{O_2} is kept constant while measuring the ventilatory response to CO_2 the hypoxic CO_2 response curve will during hypoxia be shifted towards a lower P_{CO_2} and the slope will be increased com-

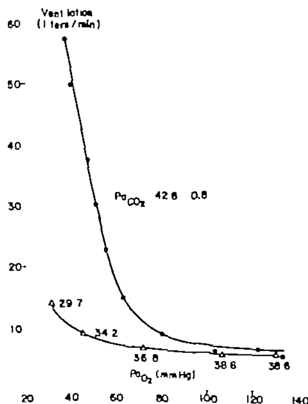


Fig. 4 Ventilatory response to hypoxia in one human subject. The upper curve shows the ventilatory response when alveolar P_{CO_2} was kept constant at 42.6 mm Hg by adding CO_2 to the inspired gas mixture. The lower curve *shows the ventilatory response* when P_{CO_2} was allowed to fall with hyperventilation. The numbers beside the lower curve refer to the measured alveolar P_{CO_2} at each point on the curve (Redrawn from Loeschcke and Gertz 1958).

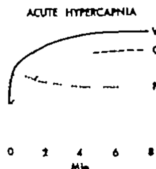


Fig 6. Diagrammatic illustration of the acute changes in peripheral (P) and central (C) chemical drive to ventilation and in ventilation (V) when Pco in inspired air is increased

results obtained in the awake human, where keeping the arterial pH constant during CO_2 inhalation removes one-fourth to two-fifths of the ventilatory response to CO_2 (Katsaros et al. 1960, Lambertsen et al. 1961) A quantitative interpretation of the latter type of experiments is however dubious because the $[HCO_3^-]$ which was injected to keep arterial pH constant may also have changed $[HCO_3^-]$ in the vicinity of central chemosensitive areas (see Chapter 6)

The sensitivity of the peripheral chemoreceptors to CO_2 depends on arterial Po_2. The sensitivity to CO_2 relative to that of the central chemosensitive areas therefore also depends on arterial Po_2. The relative contribution of the peripheral chemoreceptors to the ventilatory response to CO_2 increases when the arterial Po_2 is decreased. Interaction between hypoxia and CO_2 stimuli to the peripheral chemoreceptors complicates designing a test which measures the sensitivity of the peripheral chemoreceptors to CO_2 per se, because in such a test Po_2 must be increased above the level where Po_2 and Pco_2 interact as stimuli to the peripheral chemoreceptors. Hall et al. (1967) found in the cat interaction between Po_2 and Pco_2 when inspired Po_2 was increased from 0.21 to 1 atm. but they could not demonstrate any interaction when Po_2 was increased further These results suggest that inspired Po_2 must be increased to 1 atm. to measure the sensitivity of the peripheral chemoreceptors to CO_2 independent of their sensitivity to Po_2 changes but data are only available from the cat.

We designed a single breath test to measure in man the sensitivity to CO_2 of the peripheral chemoreceptors independent of their sensitivity to hypoxia (Sorensen and Cruz 1969). The subject breathed oxygen on a circle system for at least 10 minutes prior to the test and between the tests. He then inhaled one large breath of CO_2 in O_2 and the ventilatory response was measured as volume × frequency of the second breath following inhalation of the gas mixture containing CO_2. Three different mixtures of CO_2 in O_2 were used in the test and the procedure was repeated several times with each CO_2 mixture. The Pco_2 at the mouthpiece was monitored continuously with an infrared CO_2 analyzer and a CO_2 response curve was obtained by plotting ventilation (the second breath) as a function of "alveolar" Pco_2 during the first expiration following inhalation of the CO_2 containing mixture (see p 30 and p. 31)

A single breath test to measure the peripheral chemoreceptor sensitivity to CO_2 is based on the difference in time required for CO_2 to affect the peripheral and central

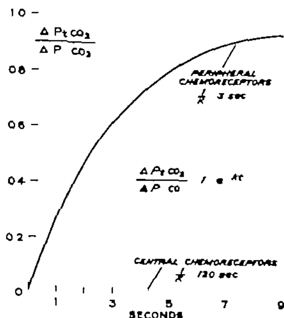


Fig. 7 Timecourse of changes in P_{CO_2} in peripheral chemoreceptors and in central chemosensitive areas after a square wave increase in arterial P_{CO_2}. The calculation is based on assumed blood flows of 2000 ml/100 g × min and 50 ml/100 g × min to the peripheral chemoreceptors and central chemosensitive areas respectively and a 3 sec difference in mean circulation time from the lung to the two areas (From Sørensen and Cruz 1969).

chemosensitive areas (Lambertsen, Gelfand and Kemp 1965). The difference in blood flow to the two areas is very large, wherefore the rate of rise in P_{CO_2} in the peripheral chemoreceptors after an increase in arterial P_{CO_2} far exceeds the rate of rise in P_{CO_2} in the central chemosensitive areas (Fig. 7) However the changes in P_{CO_2} in peripheral chemoreceptors and central chemosensitive areas shown in fig. 7 reflect the change in stimulation of the two areas only when the relative effectiveness of CO_2 as stimulus to ventilation is similar in the peripheral and central chemosensitive areas. A proportionally greater sensitivity of the central chemosensitive areas to CO_2 may result in a centrally mediated response overlapping that from the peripheral chemoreceptors, which renders the method infeasable for evaluation of the sensitivity of the peripheral chemoreceptors to CO_2 when their sensitivity is low (for instance during O_2-breathing)

The stimulus to the peripheral chemoreceptors is poorly defined with the single-breath method described by Sorensen and Cruz (1969) because it does not include measurements of arterial P_{CO_2} or pH but only measurements of mouthpiece P_{CO_2} The changes in arterial P_{CO_2} immediately following a sudden change in inspired P_{CO_2} are difficult to predict from changes in alveolar P_{CO_2} The alveolo-arterial P_{CO_2}-gradient will be reversed and increased and the effect of ventilation/perfusion inequalities in the lung on the alveolo-arterial P_{CO_2}-gradient will be exaggerated. It is therefore impossible to construct a true stimulus-response curve without simultaneous arterial pH or P_{CO_2} measurements, and the method cannot be used to compare the responses between groups where differences in ventilation/perfusion inequalities are present. The method has, despite its limitations, been applied to compare the sensitivity of the peripheral chemoreceptors to CO_2 in man at sea level and in man at high altitude as will be discussed in Chapter 4

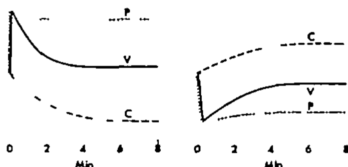


Fig. 8. Diagrammatic illustration of the acute changes in peripheral (P) and central (C) chemical drive to ventilation and in ventilation (V̇) following a sudden metabolic change in blood pH.

Metabolic acidosis and alkalosis Interaction between peripheral and central chemosensitive areas also determines the magnitude of the ventilatory response to acute metabolic acid-base changes in blood (Fig. 8) In metabolic acidosis the increase in ventilation due to stimulation of the peripheral chemoreceptors will be partly offset by a decrease in central drive to ventilation due to the ensuing hypocapnia (Gesell and Hertzmann 1926, Robin et al. 1958). Opposite changes will occur in metabolic alkalosis. This entails a compromise in terms of maintenance of a constant pH in different body compartments. The stimulation of the peripheral chemoreceptors by arterial pH changes tends to maintain arterial pH constant. The stimulation of central chemosensitive areas by pH changes in their environment tend to maintain pH constant in the brain. The eventual pH change in blood and brain after a certain acute metabolic acid-base change in the blood will depend on the relative sensitivity of the peripheral and central chemosensitive areas to pH changes in their environment.

Stability of brain extracellular fluid pH Several studies have during the last decade dealt with the changes in brain extracellular fluid pH (measured in cerebrospinal fluid) incurred by acute and chronic changes in arterial Po_2, Pco_2 and pH Much of the discussion has centered around the question whether cerebrospinal fluid pH changes during chronic metabolic acid-base changes in blood (Fencl Miller and Pappenheimer 1966, Fencl, Vale and Broch 1969) or whether it is maintained constant (Mitchell et al. 1965). The findings during chronic ventilatory adjustments will be the subject of the discussion in Chapter 6 We may however attempt a quantitative estimate of the changes in csf pH during acute ventilatory changes on the basis of the known interaction between peripheral and central chemosensitive areas. This will illustrate the efficiency of the ventilatory control system in maintaining a fairly constant pH in brain extracellular fluid.

The ventilatory response to changes in Pco_2 acts as a negative feedback system which modifies the ventilatory response to other ventilatory stimuli e.g. hypoxia. The ventilatory response to steady levels of increased inspired CO_2 concentrations (CO_2

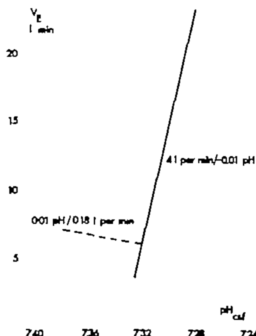


Fig. 9. Changes in ventilation as a function of csf pH when P_{CO_2} in inspired air is increased (solid line) and changes in csf pH when ventilation is increased with zero P_{CO_2} in the inspired air (dotted line). For derivation see text.

response curves) measures the sensitivity of the system to CO_2. We will assume for this calculation that the peripheral chemoreceptor contribution to the ventilatory response to CO_2 is negligible although we have previously concluded that this assumption is erroneous. We will also assume that the ventilatory response to CO_2 can be expressed as a linear function of the concommitant changes in csf pH with a slope of 4 L/min × 0.01 pH unit (Fig. 9). This value was calculated for humans by Severinghaus et al. (1963). To get a quantitative assesment of the effectivenes of the system in counteracting other ventilatory stimuli we must know the changes in csf pH incurred by a change in ventilation with zero inspired P_{CO_2}. Increasing the ventilation from 6 to 7 Liters/min results in approximately a 6 mm Hg fall in arterial P_{CO_2} and an increase in csf pH of approximately 0.055 or 0.05 pH unit/0.18 L/min increase in ventilation (Fig. 9). If we compare this value with the sensitivity of the chemosensitive areas to pH changes (4 L/min × 0.01 pH unit), we find that decreasing pH in csf 0.01 pH unit decreases the stimulus to the chemosensitive areas corresponding to 4 L/min. In other words a 0.01 pH unit increase in csf pH will impose a negative signal which is 4/0.18 or 22 times larger than the stimulus which would increase ventilation 0.18 L/min if not counteracted by the fall in P_{CO_2}. If we use an electrical analogue to the system we can call this factor the gain of the negative feed back loop.

The negative feed back loop can be blocked if we prevent P_{CO_2} from falling when we impose another ventilatory stimulus. This is done for instance when the ventilatory response to acute hypoxia is measured while keeping P_{CO_2} constant by adding CO_2 to the inspired air. In normal sea level man ventilation increases approximately 18

L/min if arterial P_{O_2} is lowered to 40 mm Hg while arterial P_{CO_2} is kept constant (Sorensen and Severinghaus 1968a, b) However if P_{CO_2} is allowed to fall during hypoxia the increase in ventilation will be only approximately 1/22 of the "iso-capnic" ventilatory response, in this case approximately 0 45 L/min. The eventual change in csf pH will be approximately 0 025

This is indeed a very crude estimate, but it indicates the magnitude of changes in csf pH to be expected when a strong ventilatory stimulus is imposed, and it illustrates that it is an inherent characteristic of the ventilatory control system to maintain pH in brain extracellular pH fairly constant. We shall in Chapter 6 discuss how this tendency to keep csf pH fairly constant is often further accentuated during ventilatory *adjustments to chronic changes in arterial* P_{O_2} P_{CO_2} *and pH*

Ventilatory Response to Acute Hypoxia in Sea Level Natives* and in High Altitude Natives*

Dripps and Comroe (1947) showed that the ventilatory responses to acute hypoxia are highly variable within a group of normal subjects and in fact some of their subjects did not increase ventilation even when the inspired O_2 concentration was lowered to 10 / (at sea level) Chiodi (1957 1963) later demonstrated that man who is born and has been living at high altitude (high altitude natives) in particular shows a blunted ventilatory response to acute hypoxia compared with the response in sea level natives somourning at high altitude. This finding was confirmed by several groups of investigators studying both Andean and Himalayan high altitude natives and using different technique to measure the ventilatory response to acute hypoxia (Severinghaus, Bainton and Carcelen 1966 Milledge and Lahiri 1967 Lefrancois, Gautier and Pasquis 1968, Sorensen and Severinghaus 1968a)

In most of these studies the ventilatory responses in high altitude natives were compared with that of sea level natives during a short sojourn at high altitude and it could not be resolved whether the blunted response in high altitude natives is a genetic characteristic of the high altitude natives or whether it is related to the duration of the hypoxic exposure. Sorensen and Severinghaus (1968a) therefore determined whether sea level natives who had lived many years at high altitude (4300 m) showed a decreased ventilatory response to acute hypoxia. The ventilatory response to acute hypoxia (ΔV_{40}) was measured from hyperoxic and hypoxic CO_2 response curves at a constant P_{O_2} = 150 mm Hg and 40 mm Hg) (see p 16). In fig. 10 the ventilatory response to acute hypoxia in a group of sea level natives is plotted as a function of duration of stay at altitude These subjects had all arrived at high altitude as adults. There is not any suggestion of a decrease in the ventilatory response to hypoxia with time at high altitude, suggesting that sea level natives do not develop a blunted response to hypoxia during prolonged exposure to hypoxia in adult life.

The study might be criticized on the grounds that the subjects who remain at high altitude for a long time might represent a selected group with respect to ventilatory response to acute hypoxia. In particular a selection of this kind might be suspected if the development of secondary illnesses, like chronic mountain sickness, is related to the ventilatory response to acute hypoxia. There is no evidence that such a selection takes place, but it will be necessary to make repeated measurements in sea level natives during prolonged sojourn at high altitude in order to answer definitely if a sea level native might develop a blunted response when exposed to hypoxia for many years during the adult life.

* The use of th term *native* refers to the place of b rth

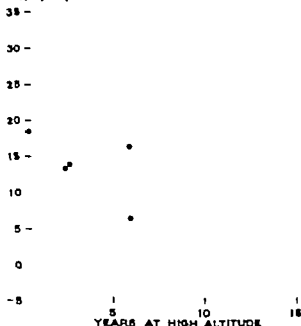


Fig. 10. Ventilatory response to acute hypoxia (ΔV_{40}) in sea level natives sojourning at an altitude of 4300 m. There is no tendency to a decrease in ΔV_{40} with duration of stay at high altitude (From Sorensen and Severinghaus 1968)

The low ventilatory response to acute hypoxia in the high altitude native is an irreversible phenomenon as shown by Sorensen and Severinghaus (1968b). We studied Andean high altitude natives who had been sojourning at sea level for many years and found that there was no tendency to an increase in response with time at sea level (fig. 11). This finding was later confirmed by Lahiri et al (1969). Several of the subjects had only spent the early years of childhood at high altitude (Fig. 12) which in connection with the findings in adult sea level natives sojourning at high altitude suggested that the ventilatory response to acute hypoxia might be irreversible determined during early childhood by the P_{O_2} in the environment. The other possibility was that a genetic factor plays a role in determining the response. This latter pos-

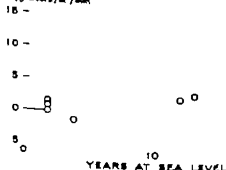


Fig 11 Ventil tory response t acute hypoxia (ΔV_{40}) in high altitude natives sojo rni g t sea level. There is no tendency to an increase i ΔV_{40} with duration of stay at sea level (From Sorensen nd Severinghaus 1968 b)

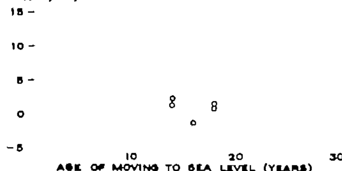


Fig. 12. Ventilatory response to acute hypoxia (ΔV_{40}) i high altitud natives sojourning at sea level (the same subjects as shown in fig 11) ΔV_{40} is low even in high altitud tives who spent only their early childhood at high lti tude (From Sorensen a d Seve ri ghaus 1968 b)

sibility is appreciated when considering that a low ventilatory response to acute hypoxia at that time had been demonstrated only in the Andean native and in the Himalayan Sherpa. Both groups are of the Mongol race with little Caucasian ad mixture, whereas most of the sea level natives studied were of the Caucasian race. In order to distinguish between the role of heritage and the role of early childhood hypoxia it was necessary to find a group of subjects who were genetically similar to the sea level natives and who had been exposed to chronic hypoxia during the earliest childhood Children born with cyanotic heart diseases offered this opportunity

It is difficult to measure the ventilatory response to acute hypoxia in a subject who has a low arterial Po_2 due to a veno-arterial shunt because a change in alveolar Po_2 is reflected to a lesser extent in the arterial blood. If the patients who were born with cyanotic heart disease were similar to the high altitude natives they would however show a blunted ventilatory response to acute hypoxia even after the hypoxemia was relieved by surgical correction of the heart disease. Sorensen and Severinghaus (1968 c) studied five young adults some years after their cyanotic heart disease (tetralogy of Steno-Fallot) had been finally corrected and found that they also showed a blunted ventilatory response to acute hypoxia (Fig. 13) These subjects were all Caucasians indicating that the low ventilatory response to hypoxia in high altitude natives is not genetically determined but rather caused by exposure to chronic hypoxia during infancy*).

None of these studies indicate if the neo-natal period is the critical time for the development of a low ventilatory response to hypoxia because both the high altitude natives studied at sea level and the patients with cyanotic heart disease had been hypoxic for some years during infancy Neither did any of the studies indicate where the "lesion" responsible for the low response is located anatomically It could be either in the peripheral chemoreceptors or in the central integrative mechanism in the respiratory center

*) Additional supportive evidence for this conclusion has recently been btained by Forster et al. (personal communicati) They found that sea level tives f European descent wh bega residence t 3100 m altitude d ri g childhood also have reduced sensit vity to hypoxia.

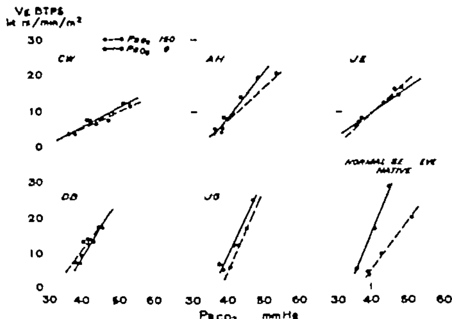


Fig. 13 Ventilatory response to CO t high nd low arterial Po_2 I 6 subjects who were born with cyanotic heart disease (tetralogy f Steno-Fallot) but who had their heart disease surgically corrected prior to the study The CO_2 response curves in ne normal subject re bown f comparison (From Sorensen nd Severi ghaus 1968c)

The elucidation of this problem would be facilitated if an animal species which is readily available for experimentation also showed a blunted ventilatory response to hypoxia after prolonged exposure to hypoxia in the early part of life but until now all attempts to find an animal model have failed Brooks and Tenney (1968) compared the ventilatory response to acute hypoxia in high altitude llama with that of llama native to sea level and found no difference. Hornbein and Sorensen (1969) compared the response of cats native to an altitude of 4300 m in the Andes with that of North American cats native to sea level and found no difference. Lefrancois, Gautier and Pasquis (1968) found a considerable diminution of ventilation following two breaths of pure oxygen in dogs native to an altitude of 5200 m They did not compare the response with that of sea level dogs in the study but considered it comparable to the response in dogs at sea level (Bouverot et al. 1965) Mines and Sorensen (1969) studied two goat kids who were born and had stayed at high altitude for the first three months of their lives. They showed a marked ventilatory response to acute hypoxia comparable to that of normal adult sea level goats (Mines and Sorensen 1970) Therefore there is a definite species difference in the development of a low ventilatory response to acute hypoxia

Because attempts to find an animal model have failed we tried to define the anatomical location of the "lesion in man Recordings from single fiber preparations of the carotid sinus nerve indicate that the same fibers carry the information when the

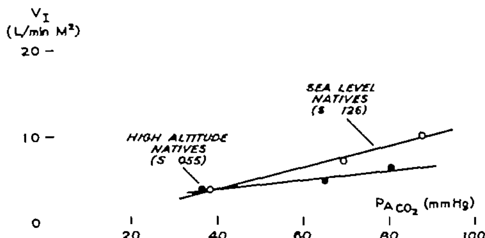


Fig. 14. Ventilatory response to a single breath of CO_2 in O_2 in 12 sea level natives and 13 high altitude natives living at sea level. Each subject was tested five times with each of three different mixtures of CO_2 in O_2. The derivation of the "CO_2 response curve" is described in Chapter 3. (Sørensen and Cruz 1969).

peripheral chemoreceptors are stimulated by hypoxia and when they are stimulated by CO_2 (Biscoe, Sampson and Purves 1967) Therefore a normal sensitivity of the peripheral chemoreceptors to CO_2 in the presence of a low sensitivity to hypoxia would indicate that the "lesion" is located in the peripheral chemoreceptors. A simultaneous low peripheral chemoreceptor mediated response to CO_2 and to hypoxia would on the other hand, be compatible with both a peripheral and a central location of the "lesion"

We attempted to measure the sensitivity of the peripheral chemoreceptors to CO_2 in man by applying the single breath CO_2 test described in Chapter 3 (Sorensen and Cruz 1969) The ventilatory response to a single large breath of CO_2 in O_2 was compared in high altitude natives and sea level natives studied at the same altitude. A group of sea level natives and a group of high altitude natives were studied at an altitude of 4300 m and other groups of sea level natives and high altitude natives were studied at sea level All the subjects had been living for a long time at the altitude where they were studied Three different mixtures of CO_2 in O_2 were used and the results were plotted as "CO_2 response curves" (Fig. 14 and 15) The construction of the CO_2 response curve is described in Chapter 3 The slope of the single breath "CO_2 response curve" is lower in the high altitude natives than in sea level natives at both altitudes

A procedure for testing the sensitivity of the peripheral chemoreceptors to CO_2 can be used to distinguish between a peripheral and central location only if the peripheral chemoreceptors are not subject to a "hypoxia" stimulus interfering with the response

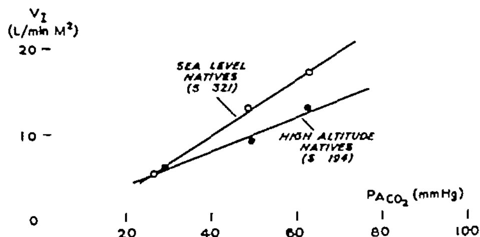


Fig. 15 Ventilatory response to a single breath of CO_2 in O_2 in 70 high altitude natives and 11 sea level natives living at high altitude (4300 m) (From Sorensen and Cruz 1969).

to CO_2 Information is not available about the Po_2 above which Po_2 and Pco_2 does not interact as stimuli in man. In normal man at sea level ventilation decreases after a single breath of oxygen indicating the presence of a Po_2 sensitive drive from the peripheral chemoreceptors in man breathing ambient air at sea level (Dejours et al. 1957) An in crease in inspired Po_2 above 1 atm might in man cause a further depression of the peri pheral chemoreceptor drive at a constant Pco_2 although Hall et al. (1967) in the cat found interaction between Po_2 and Pco_2 only when inspired Po_2 was less than 1 atm The latter information is not sufficient to validate the assumption that the ventilatory response to a single breath of CO_2 in O_2 in man measures the sensitivity of the peri pheral chemoreceptors to CO_2 independent of their sensitivity to hypoxia, and the results obtained by Sorensen and Cruz (1969) are therefore inconclusive The differ ences in the ventilatory response to a single breath of CO_2 in O_2 between sea level natives and high altitude natives might reflect only the differences in the sensitivity of their peripheral chemoreceptors to hypoxia. However if the response to CO_2 had been similar in the two groups it could have been concluded that the hypoxic lesion" is in the peripheral chemoreceptors.

Indirect evidence suggests that the high altitude natives have a normal peripheral chemoreceptor sensitivity to CO_2 despite a low sensitivity to hypoxia. When the peripheral chemoreceptors are denervated in dogs and goats resting arterial Pco_2 increases and the slope of the CO_2 response curve decreases (Bouverot et al. 1965 Mitchell 1965 Sorensen and Mines 1970) and in man arterial Pco_2 also increases when the carotid chemoreceptors are denervated as a therapeutic measure in asthmatics (Wood, Frankland and Eastcoff 1965) In normal high altitude natives studied at sea level

the resting P_{CO_2} is however similar to the P_{CO_2} in normal sea level natives and the slopes of their CO_2 response curves are not decreased suggesting that the peripheral chemoreceptor sensitivity to CO_2 is preserved (Sorensen and Severinghaus 1968b).

One might speculate about a possible mechanism for the low hypoxia sensitivity if the "lesion" is located in the peripheral chemoreceptors. Brains of mammals exposed to chronic hypoxia during infancy shows an increased vascularity following altitude hypoxia, shunt hypoxia (cyanotic heart disease) and the respiratory distress syndrome in infancy (Mercker and Schneider 1949 Mercker and Opitz 1949 Polter 1956, Cohen 1960 Rosan personal communication) If the peripheral chemoreceptors similarly shows an increased vascularity following chronic hypoxia during infancy this would result in a shorter diffusion pathway for oxygen in the chemoreceptor tissue. The mean tissue oxygen tension of the chemoreceptors would hereby be higher at any given arterial P_{O_2}. It may be difficult to demonstrate an increased vascularity in the already highly vascularized peripheral chemoreceptors, but a study of the peripheral chemoreceptor morphology in people who have died following chronic hypoxia during infancy might provide valuable information

Chronic Ventilatory Adjustments

When a mammal is exposed to hypoxia or to a change in blood acid-base composition for hours or days ventilation will change relative to the acute ventilatory response which reaches completion within minutes. The ventilatory changes during chronic hypoxia are adaptive in that they increase arterial P_{O_2} hence they have been termed ventilatory acclimatization to hypoxia The ventilatory changes associated with chronic hypoxia and chronic blood acid-base changes are closely linked together in terms of the underlying mechanisms and it is therefore appropriate to use a common term "chronic ventilatory adjustments" for all situations. Studies of the ventilatory changes during chronic hypoxia such as in man sojourning at high altitude, has been a useful tool in elucidating the mechanisms behind the chronic ventilatory adjust ments. We shall therefore use the changes in ventilation during chronic hypoxia as a starting point for the discussion.

Chronic hypoxia. When P_{O_2} in inspired air is lowered ventilation in most cases increases immediately due to stimulation of the peripheral chemoreceptors. This increase in ventilation is immediately reversed if the inspired oxygen tension is restored to normal. If a low inspired P_{O_2} is maintained for hours or days ventilation increases further and restoring the inspired oxygen tension to sea level value decreases ventilation only partially towards the prehypoxic level (Hasselbalch and Lindhard 1911 Rahn et al 1953 Kellog 1963 Severinghaus et al 1963 Severinghaus, Bainton and Carcelen 1966 Milledge and Lahiri 1967 Sorensen and Severinghaus 1968a). Therefore during chronic hypoxia ventilation is increased (and P_{CO_2} decreased) even when the hypoxic drive from the peripheral chemoreceptors is removed It should be mentioned that it has been questioned whether an increased peripheral chemoreceptor drive persists during chronic hypoxia Bjurstedt (1946) found that blocking of the nerves from the peripheral chemoreceptors did not cause a decrease in ventilation after some hours of hypoxia in the cat. This finding which suggested that the peripheral chemoreceptors adapt to hypoxia has however been contested by many investigators and it is today agreed that the hypoxic drive from the peripheral chemoreceptors persists although modified during chronic hypoxia (Åstrand 1954 Dejours et al. 1959 Ceretelli 1961 Tenney Remmers and Mithoefer 1963, Severinghaus, Bainton and Carcelen 1966 Sorensen and Severinghaus 1968a).

A qualitative description of the changes in peripheral and central chemoreceptor drive and in ventilation during acute and chronic hypoxia is shown in fig. 16. The acute ventilatory response is associated with an increase in peripheral drive and a decrease in central chemoreceptor drive to ventilation as described in Chapter 3 During chronic hypoxia ventilation increases despite a decreasing peripheral che

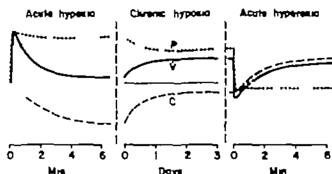


Fig. 16 Diagrammatic illustration of the changes in peripheral (P) and central (C) chemical drive to ventilation and in ventilation ($\dot{V}$) during acute and chronic hypoxia and when inspired P_{O_2} is suddenly restored to sea level values during chronic hypoxia.

moreceptor drive The change in peripheral chemoreceptor drive reflects the effect of an increase in P_{O_2} which is partly counteracted by renal compensation of the respiratory alkalosis. If the inspired P_{O_2} is restored to a normal sea level value during chronic hypoxia then the peripheral chemoreceptor drive decreases immediately to a subnormal value due to the respiratory alkalosis which is not completely corrected even within a three weeks sojourn at high altitude (Kellog 1963) Ventilation is however still increased above the prehypoxia level indicating that the drive from the central chemosensitive areas must be increased.

This qualitative description does not ignore the role of blood acid base changes in the ventilatory adjustments during chronic hypoxia but ample evidence substantiates that the increase in ventilation during acclimatization to chronic hypoxia cannot be explained by the concommitant blood acid base changes (Nielsen and Smith 1952, Kellog 1963 Severinghaus et al. 1963).

This description implies that the central chemoreceptor drive to ventilation increases during acclimatization despite a falling P_{CO_2} The ventilatory drive from the central chemosensitive areas may be related to the changes in pH of the fluid surrounding the central chemosensitive areas (see Chapter 2). The pH in brain extracellular fluid is described by the Henderson Hasselbalch equation

$$pH = pk + \log \frac{[HCO_3^-]}{S \times P_{CO_2}}$$

where pk is the negative exponent of the apparent first dissociation constant of H_2CO_3 in brain extracellular fluid and S is the solubility coefficient for CO_2

Because ventilation is stimulated by a lowering of pH in brain extracellular fluid, the increase in the central chemical drive despite a fall in P_{CO_2} suggests that the brain extracellular fluid $[HCO_3^-]$ decreases during chronic hypoxia. It was Pauli, Vorbürger and Reubi (1962) who first demonstrated that the $[HCO_3^-]$ in brain extracellular fluid (measured in csf) indeed decreases when man ascents to high altitude but Severinghaus et al. (1963) independently obtained the same information and suggested an explanation for the fall in $[HCO_3^-]_{csf}$

The hypothesis proposed by Severinghaus et al (1963) was based on the observation that H^+ and HCO_3^- is not in electrochemical equilibrium across the blood brain barrier*). At a normal arterial pH csf is a few mV positive relative to blood (Lehman and Meessman 1974 Mottschall and Loeschcke 1963 Held, Fencl and Pappenheimer 1964) and the mean capillary $[HCO_3^-]$ and pH are higher than $[HCO_3^-]_{csf}$ and pH_{csf} (Severinghaus et al. 1963) indicating that there is an electrochemical potential difference for H and HCO_3^- between csf and blood. The existence of an electrochemical potential difference may be explained by active transport processes moving either H from blood to csf or $[HCO_3^-]$ from csf to blood or alternatively by a H^+ source in the brain.

Severinghaus et al. (1963) suggested that the electrochemical disequilibrium was maintained by active ion transport and that the active ion transport was increased in hypoxia by the respiratory alkalosis resulting from hypoxic stimulation of the peripheral chemoreceptors. The chain of events were proposed to be the following 1) stimulation of the peripheral chemoreceptors, 2) hypocapnic alkalosis decreasing the central chemical drive to ventilation 3) increased transport of H from blood to csf or of $[HCO_3^-]$ from csf to blood which would restore pH_{csf} and the central chemical drive to ventilation towards normal In this mechanism hypoxic stimulation of the peripheral chemoreceptors was the essential event which initiated and maintained the lowering of $[HCO_3^-]_{csf}$ and the acclimatization induced by hypoxia would therefore be entirely dependent on hypoxic stimulation of the peripheral chemoreceptors. According to this hypothesis acclimatization to chronic hypoxia was in essence a chronic ventilatory adjustment to respiratory alkalosis resulting from peripheral chemoreceptor stimulation and an analogous explanation was indeed proposed for the chronic ventilatory adjustment during acid-base disturbances in blood (Mitchell et al. 1965).

The proposed mechanism was teleologically appealing because it would tend to maintain pH in brain extracellular fluid constant. However a number of experimental findings failed to support the hypothesis. If the hypothesis was valid two premisses had to be fulfilled 1) The absolute increase in ventilation during chronic respiratory alkalosis should be independent of a simultaneous presence or absence of hypoxia 2) During chronic hypoxia ventilation should increase only if ventilation increased acutely due to stimulation of the peripheral chemoreceptors.

If arterial P_{CO_2} is lowered for hours by mechanical hyperventilation the CO_2 response curve shifts towards a lower P_{CO_2} (Brown et al. 1948, Brown et al. 1949 Brown, Hemingway and Visscher 1950, Smith, Spalding and Watson 1962) and the $[HCO_3^-]$ in brain and csf decreases (Brown 1950, Weyne Demeester and Leusen 1968 Kjällquist, Nardini and Siesjö 1969) Eger et al. (1968) investigated whether hypoxia

* When the term "blood-brain barrier" is used i the present context it includes both the blood brain and the blood liq or barriers. In Chapter 6 we shall discuss tomical fi dings which indicate that the major resista ce to diffusion between blood nd the brai compartments is across the epithelial cells in the horoid plexus and across the endothelial cells in brain.

influenced the shift of the CO_2 response curve during hypocapnia in man by comparing the shift of CO_2 response curves measured after P_{CO_2} had been kept at different levels for 6 hours by hyperventilation with and without simultaneous hypoxia. They found that the CO_2 response curve measured after the six hour period was shifted towards a lower P_{CO_2} when hypoxia was superimposed on a certain degree of hypocapnia during the six hour period. They interpreted their results as indicating that hypoxia merely accelerates the shift of the CO_2 response curve due to hypocapnia and they suggested that the eventual shift after more prolonged hypocapnia would be the same in normoxia and hypoxia in accordance with the scheme proposed by Severinghaus et al. (1963) We might however interpret their results as indicating that hypoxia increases ventilation during sustained hypoxia also by a mechanism which is unrelated to the hypocapnia produced by hypoxic stimulation of the peripheral chemoreceptors. We shall later discuss how the latter interpretation is substantiated by the demonstration of a decrease in $[HCO_3^-]_{csf}$ during iso-carbic hypoxia (Mines and Sørensen 1971) but we shall first examine if the second premise in the hypothesis by Severinghaus et al. (1963) is fulfilled

Since the peripheral chemoreceptors were discovered it has been debated whether ventilation is stimulated during hypoxia when the peripheral chemoreceptors are removed or denervated. In the absence of peripheral chemoreceptors ventilation is initially depressed when P_{O_2} in the inspired air is lowered (Gemmill and Reeves 1933 Mitchell 1965 Sorensen and Mines 1970) Moyer and Beecher (1942) and Davenport et al. (1947) who studied anesthetized and unanesthetized chemoreceptor denervated dogs respectively found, however a delayed hyperventilation which persisted for some time after inspired P_{O_2} had been restored to normal but Davenport et al. (1947) observed mainly tachypnea which may have been due to discomfort in the awake dog. Also the time course of the ventilatory changes was fairly rapid (minutes) which distinguished these phenomena from the changes in ventilation observed during chronic hypoxia. It is possible, though, that the findings are explained by the mechanisms which are also responsible for the ventilatory changes during chronic hypoxia. Other did not demonstrate any hyperventilation during hypoxia in chemoreceptor denervated animals (Gemmill and Reeves 1933 Watt, Dumke and Comroe 1943)

Only two studies have earlier dealt with ventilatory changes during chronic high altitude hypoxia (days) in animals deprived of their peripheral chemoreceptors. Decharnaux (1934) studied two dogs who were brought to an altitude of 2300 m. He did not measure ventilation but judged their changes in ventilation from the changes in CO_2 and O_2 concentrations in blood. Very small differences were revealed between sea level and high altitude but he concluded that the animals hyperventilated at altitude. The opposite conclusion was reached by Gilfillan et al (1958) but their results have still only been published in an abstract. Thus, available evidence was not sufficient to determine if animals deprived of the peripheral chemoreceptors hyperventilate when exposed to hypoxia for prolonged periods of time.

Other findings suggested that man hyperventilates at high altitude even in the absence of a peripheral chemorecept mediated hyperventilation. The ventilatory

response to acute hypoxia is highly variable in normal man and some may not hyperventilate even at low inspired oxygen tensions (Dripps and Comroe 1947 Sorensen and Severinghaus 1968a), but everyone seems to hyperventilate during prolonged exposure to hypoxia at altitude and the alveolar P_{CO_2} does not vary much among individuals at any given altitude (FitzGerald 1913 Lahiri 1968) Also normal high altitude natives who show little or no ventilatory response to acute hypoxia has a lower arterial P_{CO_2} at altitude than when sojourning at sea level (Sorensen and Severinghaus 1968a,b), which suggests that a peripheral chemoreceptor mediated hyperventilation is not essential for the hyperventilation during chronic hypoxia

In order to study the role of the peripheral chemoreceptors in the mechanisms of ventilatory adjustments to chronic hypoxia Sorensen and Mines (1970) sought an animal which would show similar ventilatory changes as man during acute and chronic hypoxia. The unanesthetized goat proved to be well suited for studies of the ventilatory changes during acute and chronic hypoxia (Mines and Sorensen 1970) In awake animals as in awake man resting ventilation is difficult to measure accurately because it is often affected by the experimental situation and the effect of acute and chronic hypoxia on ventilation was therefore always expressed as the effect on the position of the CO_2 response curve.

In the normal awake goat ventilation increases immediately when inspired P_{O_2} is lowered and ventilation increases further during chronic hypoxia. In four goats the carotid bodies were denervated by sectioning the sinus nerves (Sorensen and Mines 1970). Following carotid body denervation ventilation was in two of the goats depressed immediately when the inspired P_{O_2} was lowered. These two goats were therefore used to examine whether an animal hyperventilates during chronic hypoxia when ventilation is *not increased acutely by hypoxia. The two goats were brought to an* altitude of 3800 m and the changes in ventilation were followed by measuring the ventilatory response to inhaled CO_2 (CO_2 response curves) while alveolar P_{O_2} during the measurements was increased to about 200 mm Hg both at sea level and at high altitude.

When the ventilatory response CO_2 is measured during acute hyperoxia the acute effects of hypoxia on ventilation are reversed and the change in position of the CO_2 response curve is therefore primarily a function of the change in brain extracellular fluid [HCO_3^-] if we assume that the central chemosensitive areas sense extracellular fluid [H]. The effect of chronic hypoxia on the position of the hyperoxic CO_2 response curve is depicted in Fig. 17 as the change in P_{CO_2} at a reference ventilation which was chosen on the basis of the animal's weight) Both goats hyperventilated at high altitude indicating that hypoxic stimulation of the peripheral chemoreceptors is

This method for quantitating the shift f the CO_2 response rve is based on the method suggested by Kellog et al. (1957) which was introduced t void the uncertainty bout the exact positio f the lower portio of the CO_2 response curve. I ou experiments the CO_2 response curve was defined as the linear regression line f the measured points and the choice of the refer ence ventilation is theref re arbitrary

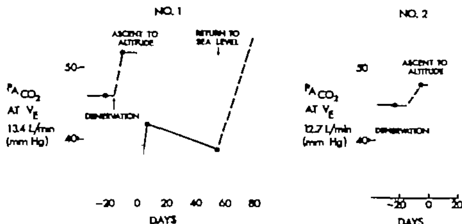


Fig. 17 Changes in the position of CO_2 response curves in two goats after carotid chemoreceptor denervation and during chronic hypoxia. The position of the CO_2 response curve is expressed as the P_{CO_2} required to drive ventilation to a given value which was chosen on the basis of the animal's weight. The CO_2 response curves were both at sea level and at high altitude measured after the alveolar P_{O_2} had been acutely increased to about 200 mm Hg (From Sorensen and Mines 1970).

not essential for the hyperventilation at high altitude. The small sample and the fact that the aortic chemoreceptors were left intact made it however desirable to reexamine the question in an animal deprived of both carotid and aortic chemoreceptors. Rabbits were used (Sørensen 1970) because both aortic and carotid chemoreceptors can be denervated on the neck thereby avoiding intrathoracic surgery The rabbit is not very cooperative in respiratory studies while awake and we therefore instead used the change in $[HCO_3^-]_{csf}$ as a measure of the ventilatory changes during chronic hypoxia. The effect of 24 hours hypoxia (P_B = 470 mm Hg) on $[HCO_3^-]_{csf}$ in normal and chemoreceptor denervated rabbits is shown in fig. 18 Hypoxia caused a fall in $[HCO_3^-]_{csf}$ in both intact and chemoreceptor denervated animals. The mean changes in $[HCO_3^-]$ during hypoxia were actually the same in the two groups of animals which may be tortuitous. These results agree with our findings in goats and indicate that the ventilatory adjustments during chronic hypoxia does not require the presence of intact peripheral chemoreceptors. It should be noted that P Bouverot (personal communication) also found that one chemoreceptor denervated dog hyperventilated when brought to high altitude.

It must be emphasized that these findings do not exclude the possibility that an intact peripheral chemoreceptor drive might be advantageous to a mammal during chronic exposure to hypoxia. It only demonstrates that an animal will hyperventilate at high altitude even in the absence of peripheral chemoreceptors. These findings obviously do not contest the belief that a lowering of brain extracellular fluid $[HCO_3^-]$ is instrumental in the hyperventilation during chronic hypoxia (Severinghaus et al. 1963) but the question is How is brain extracellular fluid $[HCO_3^-]$ lowered during chronic hypoxia? Our findings can not be explained by the mechanism proposed by Severinghaus et al (1963). We have therefore suggested that an increased glycolysis

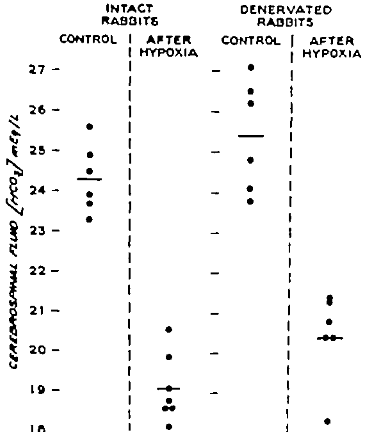


Fig. 18. The effect of 24 hours hypoxia (P = 470 mm Hg) on $[HCO_3^-]_{csf}$ in normal rabbits and in rabbits where the aortic and carotid bodies had been denervated at least a week prior to the hypoxic exposure. The completeness of denervation was ascertained by absence of a ventilatory response to intravenous NaCN. The horizontal bars indicate the mean values (From Sørensen 1970)

in the brain is the primary event which initiates and maintains the lowering of $[HCO_3^-]$ in csf at least in the denervated animals and in man without a peripheral chemoreceptor response to hypoxia (Sorensen and Mines 1970 Sorensen 1970) H formed by production of lactic and pyruvic acid in the brain will titrate $[HCO_3^-]$ in brain extra cellular fluid leading to diffusion of CO_2 and H_2O into blood.

Support for this hypothesis may be found in the work of Cohen et al (1967) in which it was observed that while less than 10 % of the brain glucose consumption is anaerobic in man breathing ambient air at sea level a lowering of arterial Po_2 to about 35 mm Hg (while keeping arterial Pco_2 constant) increases the fraction of brain glucose consumption which is anaerobic to about 25 %. In the chronically hypoxic high altitude natives living at an altitude of 4300 m we found that a similar fraction of brain glucose consumption is anaerobic when they breathe ambient air at that altitude whereas only 7 % of the glucose consumption was anaerobic after one hour of breathing oxygen (Sorensen, Milledge and Severinghaus 1969). During acute

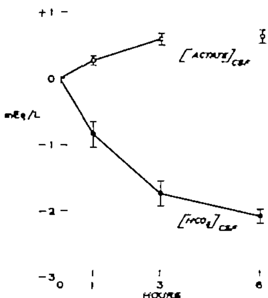


Fig. 19 Changes in [lactate]$_{csf}$ and [HCO_3^-]$_{csf}$ during six hours hypoxia in anesthetized, paralyzed dogs in which arterial pH, P_{CO_2}, and [HCO_3^-] was kept constant at a normal level during the hypoxic period. The concentration changes are expressed relative to the csf concentrations in the pre-hypoxia sample. When the animals were breathing ambient air while arterial acid-base parameters were maintained at the same level as in the hypoxia experiments csf concentrations of lactate and HCO were nearly unchanged (From Mines and Sørensen 1971).

normocapnic hypoxia cerebral blood flow increases and in the experiments reported by Cohen et al. (1967) jugular venous P_{O_2} was similar to the calculated internal jugular venous P_{O_2} of the high altitude natives breathing ambient air at 4300 m altitude (Sørensen and Milledge unpublished)

The demonstration of an increased glycolysis in the brain during acute and chronic hypoxia does not allow any conclusions about the quantitive importance of an increased H^+ production in reducing the [HCO_3^-] in brain extracellular fluid during chronic hypoxia. It will depend also on the passive flux of HCO_3^- from blood to brain extracellular fluid and/or of H^+ in the opposite direction. The effect of a certain brain H^+ production on [HCO_3^-]$_{ecf}$ will therefore depend on the permeability of the blood brain barrier to HCO_3^- and/or H^+ The minimal increases in csf lactate concentration observed in man during sojourn at high altitude (Severinghaus et al. 1963 Lahiri and Milledge 1967) has been advanced as an argument against an increased brain lactic and pyruvic acid production playing a significant role in lowering [HCO_3^-]$_{ecf}$ during chronic hypoxia and also in chemoreceptor denervated rabbits the increase in [lactate]$_{ecf}$ is small compared to the fall in [HCO_3^-]$_{ecf}$ (Sørensen 1970).

During chronic hypoxia [HCO_3^-]$_{plasma}$ decreases and because changes in [HCO_3^-]$_{pl}$ will affect [HCO_3^-]$_{csf}$ Mines and Sorensen (1971) examined if a one-to-one relationship between the change in [HCO_3^-]$_{csf}$ and the change in [lactate]$_{csf}$ would exist if [HCO_3^-]$_{pl}$ was prevented from falling during hypoxia. Anesthetized, paralyzed dogs were exposed to hypoxia for six hours while arterial [HCO_3^-], P_{CO_2} and pH were kept constant. The changes in [HCO_3^-]$_{csf}$ and [lactate]$_{csf}$ shown in fig. 19 demonstrate that [lactate]$_{csf}$ even under these circumstances increased only about one third as much as [HCO_3^-]$_{csf}$ decreased The changes in absolute concentrations of pyruvate were insignificant in comparison (Mines and Sorensen 1971). This finding suggests that the

changes in $[lactate]_{csf}$ does not indicate how much $[HCO_3^-]_{csf}$ is lowered due to titration with H^+ formed by the brain.

There is however no reason a priori to expect a one-to-one relation between the increase in csf lactate and pyruvate concentrations and the fall in $[HCO_3^-]_{csf}$ during hypoxia even if an increased H^+ production from brain glycolysis was solely responsible for titrating $[HCO_3^-]$ out of csf. In the steady state the concentration of the anion in csf is a function of its rate of formation and the permeability of the blood brain barrier for the anion.

Before we attempt a further evaluation of the role of an increased H^+ production by the brain on the steady state distribution of $[HCO_3^-]$ between blood and brain extracellular fluid we must however consider the different factors which will affect the distribution of H^+ and $[HCO_3^-]$ between the two compartments.

The Factors which Determine the Distribution of HCO_3^- and H between Blood and Brain Extracellular Fluid+

In the normal situation the $[HCO_3^-]$ is lower and the $[H^+]$ higher in csf than in the water phase of brain capillary plasma (Severinghaus et al. 1963 Pappenheimer et al. 1965) but csf is a few mV positive relative to blood (Held, Fencl and Pappenheimer 1964) indicating that HCO_3^- and H^+ are not in electrochemical equilibrium across the blood-brain barrier The maintenance of an electrochemical potential difference for H^+ and HCO_3^- could be explained either by an active transport of H^+ or HCO_3^- between blood and csf or by continuous formation of H^+ (titrating HCO_3^-) in the brain. The principal difference between the two mechanisms is that the net flux of HCO_3^- and H^+ between blood and brain extracellular fluid is zero if the electrochemical potential difference is maintained only by an active transport system, whereas an electrochemical potential difference caused by a H^+ source in the brain implies a net ion-flux of HCO_3^- from blood to brain extracellular fluid. An active transport of H^+ *and/or HCO_3^- across the blood-brain barrier does not necessarily imply zero net flux* of the ions across all of the different cell layers which constitute the blood-brain barrier There might be an active transport across one cell layer such as the choroid plexus and a passive net flux across another cell layer such as capillary endothelium.

Regardless of which postulated mechanism is correct chronic blood acid base changes and chronic hypoxia should not alter the electrochemical potential differences for H^+ and HCO_3^- between blood and csf if 1) the energy expenditure in the active transport of H^+ and HCO_3^- across the blood-brain barrier remained constant, and 2) the permeability for H^+ and HCO_3^- remained constant, and 3) cerebral H^+ production remained constant. By examining the electrochemical potential differences for H^+ and HCO_3^- during these altered conditions we may thereby determine which factors might affect the electrochemical potential differences for these ions.

The electrochemical potential differences ($\Delta\mu$) for H^+ and HCO_3^- between csf and blood (csf-blood) may be defined

$$\Delta\mu_{H} = E + 0.061\,(pH_{csf} - pH_{plasma}) \qquad (1)$$

$$\Delta\mu_{HCO_3^-} = E - 0.061 \log \frac{[HCO_3^-]_{csf}}{[HCO_3^-]_{plasma}} \qquad (2)$$

+ The electrochemical potential difference is most frequently defined as $\Delta\mu = zEF + RT \ln C_1/C_2$ with the dimension of work/Mol (Joule/Mol) (Davson 1964 Ussing 1960). The definition used here expresses the electrochemical potential difference as "an electrical work term" (Joule/Coulomb = Volt), which is also the difference between the measured potential (E) and the equilibrium potential for the ion ($RT/zF \ln C_1/C_2$) (Katz 1966).

where E is the electrical potential difference $E_{csf} - E_{plasma}$ in Volts. In order to calculate the electrochemical potential differences during blood acid base changes and during hypoxia we thus must first know whether the potential difference (E) changes during these conditions.

The electrical potential difference csf – blood A number of investigators have reported measurements in mammals of a DC potential (E) in cerebrospinal fluid with respect to blood or extrameningeal tissue (Lehman and Meesmann 1924 Loeschcke 1956a, Tschirgi and Taylor 1958, Held Fencl and Pappenheimer 1964 Welch and Sadler 1965 Goodrich 1965 Finn et al 1968 Kjällquist 1970, Sorensen and Severinghaus 1970). All investigators but Tschirgi and Taylor (1958) found csf to be a few mV positive relative to blood at a normal arterial pH Tschirgi and Taylor's (1958) finding of csf being negative with respect to blood, is probably the result of their use of NaCl bridges at the liquid junctions with csf and blood. Because Cl and Na have different mobilities and the concentrations of Cl in csf and plasma differ more than their Na concentrations, the use of NaCl bridges leads to errors in measurement.

In rabbits, dogs and rats E increases 25 to 45 mV per pH unit decrease in plasma pH during both acute and chronic metabolic and respiratory acidosis (Loeschcke 1956b Held Fencl and Pappenheimer 1964 Goodrich 1965 Kjällquist 1970 Sorensen and Severinghaus 1970 In dogs E is related linearly to changes in plasma pH (Held, Fencl and Pappenheimer Sorensen and Severinghaus 1970). In rats a curvilinear relationship has been reported (Kjällquist and Siesjö 1967 Kjällquist 1970) but recently a linear relationship has also been found in rats (Siesjö personal communication). In cats E decreases when arterial pH is lowered (Mottschall and Loeschcke 1963 Finn et al. 1968). No information is available about the potential difference csf blood in man.

The potential difference is not affected by pH changes in csf or brain interstitial fluid if pH is changed in ventriculo-cisternal perfusion fluid (Held, Fencl and Pappenheimer 1964) or in fluid bathing the ventricular side of the choroid plexus (Welch and Sadler 1965), or if pH is changed in brain interstitial fluid by creating a cerebral acidosis with cerebral hypoxia (Sorensen and Severinghaus 1970) The potential is affected by changes in [K] in ventriculo-cisternal perfusion fluid and in fluid bathing the ventricular side of the choroid plexus (Held, Fencl and Pappenheimer 1964 Welch and Sadler 1965) but it changes less when plasma [K+] is changed (Held, Fencl and Pappenheimer 1964 Bradbury and Kleeman 1967 Cameron 1971). Because [K] is maintained very constant in brain extracellular fluid (Cserr 1965 Bradbury and Kleeman 1967 Cohen, Gershenfeld and Kuffler 1968), the effect of change in [K] on the brain side of the blood brain barrier is probably of little importance during physiological conditions. E may therefore during acid base changes in blood be described by

$$E = F(pH_{pl} - pH_O) \qquad (3)$$

where pH_O is the pH_{pl} at which the potential is zero and F is the slope of the curve relating E to plasma pH

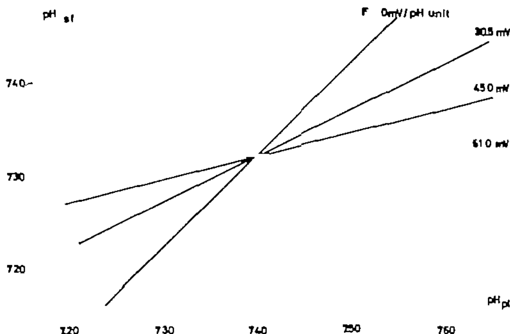


Fig. 20 Predicted relationship between pH_{csf} and pH_{pl} if $\Delta\mu_H$ is maintained consta t (although different from zero) during blood acid base changes. The predicted relationship is calculated for different values f F (see Eq (3))

Are the electrochemical potential differences for H^+ and HCO_3^- between blood and csf affected by acid-base changes in blood? From equations (1), (2) and (3) we can predict the changes in pH_{csf} and $[HCO_3^-]_{csf}$ during respiratory and metabolic blood acid-base changes if the electrochemical potential differences between blood and brain extracellular fluid remained constant (although different from zero) during acid base changes in blood. If the electrochemical potential differences for H^+ and HCO_3^- between csf and blood remain constant in two different blood acid-base situations denoted as and we can write

$$E + 61(pH_{pl} - pH_{csf}) = E + 61(pH_{pl} - pH_{csf}) \quad (4)$$

or

$$E - E = 61(pH_{pl} - pH_{pl}) - 61(pH_{csf} - pH_{csf}) \quad (5)$$

We can substitute for E using equation (3)

$$F(pH''_{pl} - pH_{pl}) = 61(pH_{pl} - pH_{pl}) - 61(pH_{csf} - pH_{csf}) \quad (6)$$

or

$$pH_{csf} - pH_{csf} = \left(1 - \frac{F}{61}\right)\left(pH_{pl} - pH_{pl}\right) \quad (7)$$

This relationship is depicted in fig. 20 for various values of F The larger F is the less will pH_{csf} change for a given change in pH_{pl}

From equation (7) we can also predict the changes in $[HCO_3^-]_{csf}$ if $\Delta\mu_{H^+}$ and

$\Delta\mu_{HCO_3^-}$ remain constant while $[HCO_3^-]_{pl}$ is changed but P_{CO_2} maintained constant We can substitute for pH using the Henderson Hasselbalch equation

$$pH = pK + \log [HCO_3^-] - \log (S \quad P_{CO_2}) \tag{8}$$

Substituting in equation (7)

$$\log [HCO_3^-]'_{csf} - \log [HCO_3^-]_{csf} = \left(1 - \frac{F}{61}\right) \left(\log [HCO_3^-]'_{pl} - \log [HCO_3^-]_{pl}\right) \tag{9}$$

or

$$\frac{[HCO_3^-]'_{csf}}{[HCO_3^-]_{csf}} = \left(\frac{[HCO_3^-]'_{pl}}{[HCO_3^-]_{pl}}\right) \left(1 - \frac{F}{61}\right) \tag{10}$$

If $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ remained constant during chronic blood acid-base changes then the relationship between changes in pH_{pl} and pH_{csf} would be a function of F only (see Eq 7). In fig. 21 we have plotted the changes in pH_{pl} and pH_{csf} measured by various investigators during chronic blood acid base changes in humans. The theoretical curves are the ones shown in fig. 20 pH_{csf} does not fall along any particular F-line" suggesting that $\Delta\mu_{H^+}$ does not remain constant during blood acid base changes.

This conclusion does not depend on the accuracy of the absolute pH values reported by the different investigators but only on the accuracy by which the difference in pH between the various conditions has been determined This eliminates an important source of error when comparing pH values measured in different laboratories.

Factor affecting the electrochemical potential differences for H and HCO_3^- between blood and csf during chronic blood acid-base changes It has been suggested that $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ between blood and csf are determined by plasma pH or $[HCO_3^-]$ (Pappenheimer et al. 1965 Fencl Miller and Pappenheimer 1966) or by pH_{csf} (Severinghaus et al. 1963 Mitchell et al 1965) Recently it was proposed that P_{CO_2} is the single factor which determines the values of $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ during chronic blood acid base changes (Siesjö and Kjällquist 1969 Kjällquist 1970). The validity of these proposals can be tested by calculating the $\Delta\mu_{H^+}$ values during blood acid base changes and examine if they correlate with any of the mentioned parameters.

The electrical potential difference between csf and blood has not been measured in humans, but we can calculate $\Delta\mu_{H^+}$ from the large number of human clinical and experimental data if we assume that the effect of plasma pH changes on the electrical potential differences between csf and blood in humans are similar to those found in dogs and rats during acute and chronic blood acid base changes (Held, Fencl and Pappenheimer 1964 Goodrich 1965 Kjällquist 1970, Sorensen and Severinghaus 1970). This may not be an entirely valid assumption but it allows us to make a qualitative estimate of the relation between the calculated $\Delta\mu_{H^+}$ and certain parameters. Our conclusions may then be compared with the conclusions which have been obtained from the few studies in animals where $\Delta\mu_{H^+}$ has been calculated from measured potentials.

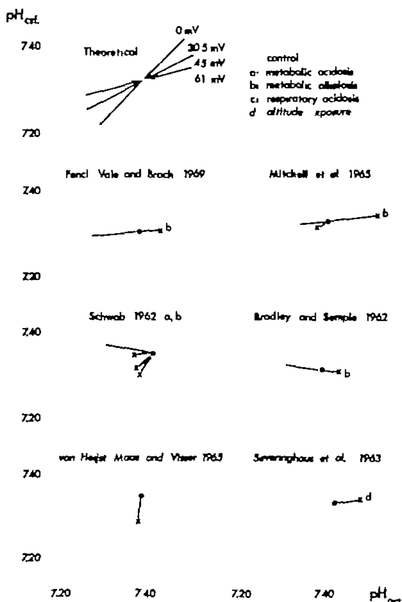


Fig. 21 Measured pH values i csf nd plasma d ring clinical and experimental chronic blood acid-base changes in humans. The theoretical curves show th predicted relationship f different values of F if $\Delta\mu_{H^+}$ remain constant during blood acid-base changes (see also fig. 20).

In fig. 22 calculated $\Delta\mu_{H^+}$ values are plotted as a function of pH_{pl} and pH_{csf} during clinical and experimental chronic blood acid base changes in humans and in dogs and rats. $\Delta\mu_{H^+}$ between csf and blood is calculated according to eq (1) p. 51 assuming E = 4 mV at pH_{pl} = 7 40 and F = 30.5 mV/pH unit). Arterial pH is

A value of 30.5 mV/pH unit was chosen because it is close to the average value found in dogs nd rats (Held, Fencl nd Pappenheimer 1964 Sorensen and Severi ghaus 1970 Kjällquist 1970).

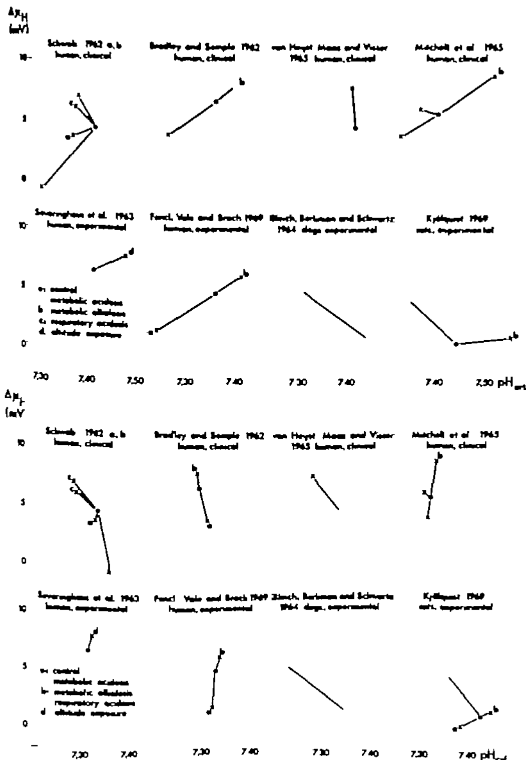


Fig. 22. Calculated changes in $\Delta\mu_{H^+}$ during various clinical and experimental chronic blood acid-base changes in humans and animals plotted as function of measured pH values in plasma csf. For calculations of $\Delta\mu_{H^+}$ see text (From Mines, Morrill and Sørensen 1971)

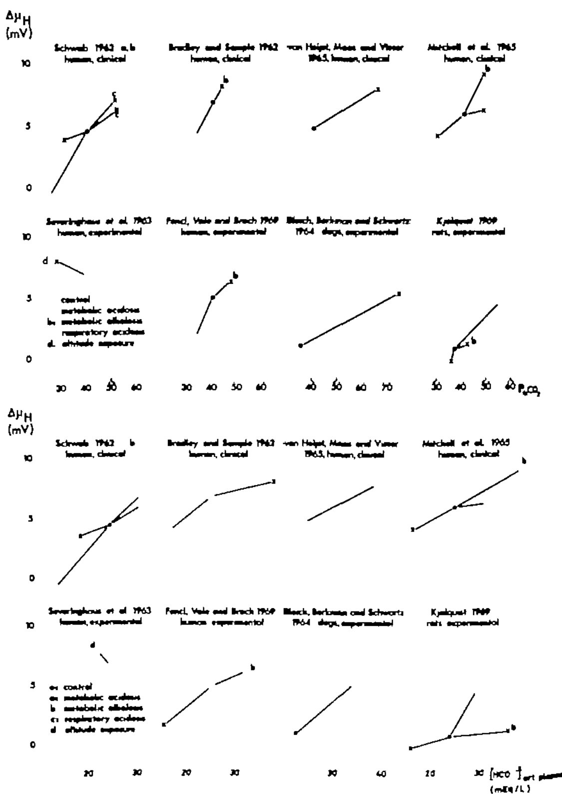


Fig. 23 Calculated changes I $\Delta\mu_H$ during various clinical a d experimental chronic blood acid-base cha ges in humans and nimals plotted as function of measured arterial P_{CO_2} a d $[HCO_3^-]$ lues. For calculations f $\Delta\mu$ see text (From Mines, Morrill and Sorensen 1971).

used as the blood pH value Only the $\Delta\mu_{H^+}$ values from Kjällquist (1970) were calculated by the author There is no apparent correlation between the changes in either pH_{pl} or pH_{csf} and $\Delta\mu_{H^+}$ suggesting that none of these two factors is the single determinant for the changes in $\Delta\mu_{H^+}$ during blood acid base changes.

If as proposed by Severinghaus et al (1963) and Mitchell et al (1965) pH_{csf} is regulated by adjustments of active transport of HCO_3^- or H^+ between blood and csf then $\Delta\mu_{H^+}$ should increase when pH_{csf} increases and decrease when pH_{csf} decreases. The absence of a consistent positive correlation between the calculated $\Delta\mu_{H^+}$ and pH_{csf} suggests that such a mechanism is not functioning Other evidence also fails to support the existence of such a mechanism (Sorensen and Mines 1970 Sorensen 1970, Mines and Sorensen 1971).

If we instead plot $\Delta\mu_{H^+}$ as a function of arterial P_{CO_2} or $[HCO_3^-]$ (Fig. 23) we find a positive correlation with both parameters during all conditions except chronic hypoxia at high altitude when there is an inverse correlation between $\Delta\mu_{H^+}$ and P_{CO_2} and $[HCO_3^-]_{pl}$. The plot suggests that P_{CO_2} and/or $[HCO_3^-]_{pl}$ are more important than either pH_{pl} or pH_{csf} in determining the magnitude of $\Delta\mu_{H^+}$ It does not however exclude the possibility that both pH_{pl} and pH_{csf} might affect $\Delta\mu_{H^+}$ between csf and blood. The inverse relation between the calculated $\Delta\mu_{H^+}$ and P_{CO_2} and $[HCO_3^-]_{pl}$ during chronic hypoxia at high altitude may be explained by the effect of hypoxia on $\Delta\mu_{H^+}$ (see p 51 and Mines and Sorensen 1971).

From the plot in fig. 23 we cannot distinguish between the relative effects of P_{CO_2} changes and $[HCO_3^-]_{pl}$ changes on $\Delta\mu_{H^+}$ during blood acid-base changes. Kjällquist (1970) found no correlation between $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$ and $[HCO_3^-]_{pl}$ when examining combinations of metabolic and respiratory blood acid-base changes, but he found a positive correlation with P_{CO_2} during all conditions. He therefore concluded that P_{CO_2} is the single factor which affect $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$ during blood acid base changes (see also Siesjö and Kjällquist 1969).

Mines, Morrill and Sorensen (1971) examined in dogs the effect of a lowering of plasma pH and $[HCO_3^-]$ while keeping P_{CO_2} constant on $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$ and found that both decreased compared with control experiments in which arterial pH P_{CO_2} and $[HCO_3^-]$ were kept at a normal level (Fig. 24). Because of the lack of a consistent correlation between changes in $\Delta\mu_{H^+}$ and pH_{pl} during metabolic and respiratory acidosis and alkalosis (Fig. 22) we concluded that $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO^-}$ is affected by changes in $[HCO_3^-]_{pl}$ which is contrary to the conclusion arrived at by Siesjö and Kjällquist (1969). In our experiments P_{CO_2} was maintained nonstant in arterial blood and csf and therefore we cannot evaluate if P_{CO_2} might also have an effect on $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$

The discrepancy between the conclusions drawn by Siesjö and Kjällquist (1969) and those arrived at by Mines, Morrill and Sorensen (1971) cannot be resolved on the basis of available information. Mines, Morrill, and Sorensen (1971) calculated $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$ assuming that E changes 30.5 mV per pH unit change in arterial pH. The changes in csf pH and $[HCO_3^-]$ during 6 hours of metabolic acidosis could how

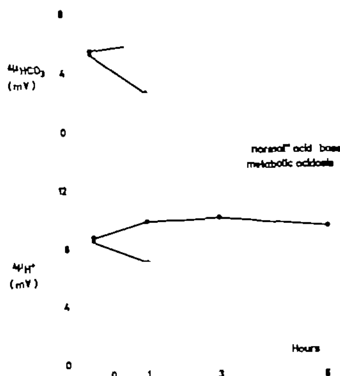


Fig. 24 Calculated electrochemical potential differences ($\Delta\mu$) for H and HCO between blood and csf during "normal" acid-base and metabolic acidosis experiments in dogs. The values are calculated assuming that the potential difference csf-blood increases 30.5 mV/pH unit (From Mines, Morrill and Sorensen 1971).

ever be explained without invoking a change in $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO^-}$ if E increased more than 30.5 mV/pH unit.

In fig. 25 the 6 hours values in arterial blood and csf measured during isocarbic metabolic acidosis is compared with the 6 hour values measured in experiments where arterial pH and [HCO_3^-] was kept at a "normal" level for 6 hours while P_{CO_2} in arterial blood and csf was the same in both types of experiments. If $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ had remained constant while E changed 30.5 mV per pH unit then the csf values during acidosis should fall along the solid line. The broken lines connecting the experimental points show the predicted relationship if $\Delta\mu_{H^+}$ and Δu_{HCO^-} remained constant while E changed 53 4 mV/pH unit and 51 8 mV/pH unit respectively Our results could therefore be explained without invoking a change in $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO^-}$ if E changed 52–53 mV/pH unit.

The $\Delta\mu$ values calculated by Kjällquist (1970) are based on a curvilinear relationship between E and arterial pH They found that the change in E per unit change in pH was greater at an alkaline than at an acid arterial pH (Kjällquist and Siesjö 1967 Kjällquist 1970). If one instead assumes a linear relationship similar to that found in dogs (Held, Fencl and Pappenheimer 1964 Sorensen and Severinghaus 1970) then $\Delta\mu$ values calculated from their data will also correlate with arterial [HCO_3^-] during metabolic and respiratory blood acid base changes, but it does not explain the lack of a correlation during the various combinations of respiratory and metabolic acid base changes which they also examined.

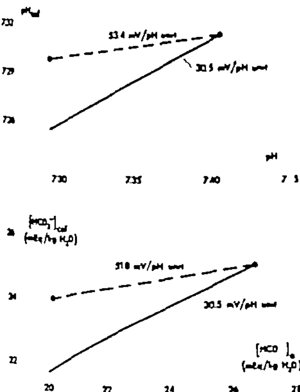


Fig. 25 Arterial pH and [HCO$_3^-$] and csf pH and [HCO$_3^-$] during metabolic acidosis and during normal acid base experiments in dogs. The solid points depict the 6 hour values in the two types of experiments. The solid line depicts the predicted relationship between the two parameters if $\Delta\mu_{H}$ and $\Delta\mu_{HCO}$ stayed constant while the potential difference csf-blood increases 20.5 mV pH unit. The broken lines describe the relationship between the two parameters if $\Delta\mu_{H}$ and $\Delta\mu_{HCO}$ stayed constant, while the potential difference csf-blood increased as much as denoted on the respective figures (From Mines, Morrill and Sorensen 1971)

We must therefore conclude that it is possible that both changes in P_{CO_2} and in arterial [HCO_3^-] affects $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$

The effect of hypoxia on the electrochemical potential differences for H^+ and HCO_3^- between csf and blood. Mines and Sorensen (1971) examined the effect of hypoxia per se on $\Delta\mu_{HCO}$ between csf and blood by exposing anesthetized paralyzed dogs to hypoxia for six hours while keeping arterial P_{CO_2} and pH constant at a normal level. The changes in cisternal fluid pH and [HCO_3^-] were compared with those observed in dogs which has a normal arterial P_{O_2} while arterial P_{CO_2} and pH were kept at the same level as in the hypoxia experiments. The electrical potential difference between csf and blood was constant because arterial pH was kept constant, (see eq (3) p. 53 and Sorensen and Severinghaus 1970) The change in the ratios between [HCO_3^-] in csf and plasma therefore parallel the changes in $\Delta\mu_{HCO}$. In fig. 26 $\Delta\mu_{HCO}$ is plotted for the two types of experiments demonstrating that hypoxia per se increases $\Delta\mu_{HCO}$

We propose that the effect of hypoxia on $\Delta\mu_{HCO}$ is due to an increased cerebral H^+ production through an accelerated anaerobic glycolysis during hypoxia even though [lactate]$_{csf}$ increases much less than [HCO_3^-]$_{csf}$ decreases (Mines and Sorensen 1971). This proposal is supported by demonstration of an increased anaerobic glycolysis in the human brain during acute and chronic hypoxia (Cohen et al. 1967 Sørensen, Milledge and Severinghaus 1969). We cannot exclude the possibility that the production of other organic acids increases significantly during hypoxia but at the present

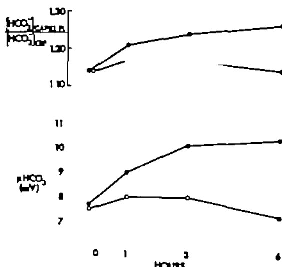


Fig. 26. Changes in the ratio of $[HCO_3^-]$ in brain capillary plasma and csf and in the calculated $\Delta\mu_{HCO_3^-}$ between csf and blood in dogs during normoxia and six hours hypoxia with constant arterial pH, P_{CO_2}, and $[HCO_3^-]$ (From Mines and Sørensen 1971).

time lactic and pyruvic acid must be considered the most probable sources of an increased H^+ production during hypoxia.

The calculated increase in $\Delta\mu_{H^+}$ during chronic hypoxia at high altitude (Fig. 23) therefore might reflect several factors which affect $\Delta\mu_{H^+}$ in opposite directions because hypoxia will tend to increase $\Delta\mu_{H^+}$ and the fall in P_{CO_2} and $[HCO_3^-]_{plasma}$ will tend to decrease $\Delta\mu_{H^+}$

The calculated increase in $\Delta\mu_{H^+}$ in patients with respiratory acidosis due to chronic lung disease may partly be explained by concommitant hypoxemia but arterial hypoxemia does not explain the calculated increase in $\Delta\mu_{H^+}$ during respiratory acidosis in dogs and rats (Bleich, Berkmann and Schwartz 1964 Kjällquist 1970) because the animals inhaled gas mixtures containing 21 % O_2.

The effect of P_{CO_2} – pH on metabolic H^+ production by the brain. Because we have postulated that the effect of hypoxia on $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO_3^-}$ is due to changes in cerebral H^+ production we should evaluate whether the positive correlation between $\Delta\mu_{H^+}$ and arterial P_{CO} and $[HCO_3^-]$ can also be explained by alterations in the H^+ production in the brain.

The effect of an increased P_{CO_2} on brain lactate and pyruvate production is not known, but when arterial P_{CO_2} is lowered to either 19 mm Hg or 10 mm Hg for 90 min in anesthetized man brain anaerobic glycolysis increases significantly (Alexander et al. 1965 Alexander et al 1968) Also during hypocarbia lactate concentration in brain and csf increases whereas it changes little during hypercarbia (Bain and Klein 1949 Plum and Posner 1967 Weyne, Demeester and Leusen 1968 Kjällquist, Nardini and Siesjö 1969). Changes in $[HCO_3^-]_{plasma}$ does not have any significant effect on brain anaerobic glycolysis (Alexander et al. 1968), which suggests that the effect of changes in $[HCO_3^-]_{plasma}$ on $\Delta\mu_{H^+}$ is not explained by metabolic events.

It may not be justified to deduce from measurements of concentration to production rates because the elimination of lactate from brain to blood may involve specialized transport mechanisms e g. facilitated diffusion implying that the concentration difference between csf and blood may not bear a constant relationship to the rate of lactate production in the brain Despite such reservations about the interpretation of concentration measurements we must however conclude that available evidence indicate that the positive correlation between arterial P_{CO_2} and $[HCO_3^-]$ and $\Delta\mu_{H^+}$ can not be explained by changes in H production from brain anaerobic glycolysis because the changes in metabolic H production are opposite to those which could explain the correlation. Because we cannot explain the changes in $\Delta\mu_H$ during blood acid-base changes by changes in cerebral H production we therefore suggest that they might be explained by interference with an active transport of H^+ and/or HCO_3^- between blood and brain extracellular fluid

The mechanisms which maintain an electrochemical potential difference for H and HCO_3^- between blood and csf during normoxia with a normal blood acid-base composition. The suggestion that the electrochemical potential differences for H^+ and HCO_3^- between blood and csf changes by interference with either one of two mechanisms (active transport of H /HCO_3^- between blood and csf and brain H production) does not indicate how much each of these mechanisms contribute to the maintenance of an electrochemical disequilibrium for H and HCO_3^- during normoxia when the blood acid-base composition is normal. Because the brain produces lactic and pyruvic acid even during normoxia (Gibbs et al 1942, Erbslöh Klärner and Bernsmeier 1958 Cohen et al. 1967) H production by the brain might explain the electrochemical disequilibrium during normoxia.

If the electrochemical potential difference for HCO_3^- is maintained by brain production of H then there must be a net flux of HCO_3^- from blood to brain extracellular fluid, which must equal the brain H production if H^+ is removed from the brain only by titrating extravascular HCO^- forming CO_2 and H_2O Therefore if the electrochemical potential difference for HCO_3^- between blood and csf was maintained only by brain H^+ production then the net flux of HCO_3^- from blood to brain should equal the brain H^+ production both during normoxia and hypoxia.

The net flux of HCO_3^- from blood to csf is a function of $\Delta\mu_{HCO}$ and the permeability of the blood brain barrier to HCO_3^- The ratio between the net fluxes of HCO_3 therefore is a function of the ratio between $\Delta\mu_{HCO}$ during normoxia and hypoxia if the permeability is the same. In the experiments by Mines and Sorensen (1971) $\Delta\mu_{HCO}$ increased about 30 / (from 7 7 mV to 10.3 mV) during isocarbic hypoxia suggesting an approximate 30 / increase in the net flux of HCO_3^- from blood to csf but the 300 / increase in brain anaerobic glycolysis which Cohen et al. (1967) observed in humans during isocarbic hypoxia is grossly different from the increase in net flux of HCO_3^- from blood to csf (comparing data from humans and dogs may not be justified).

In awake normoxic goats there is no net flux of HCO_3^- between ventriculo-cisternal perfusion fluid and surrounding tissue when the HCO_3^- in perfusion fluid is the same as the measured steady state $[HCO_3^-]$ in cisternal fluid (Pappenheimer et al. 1965

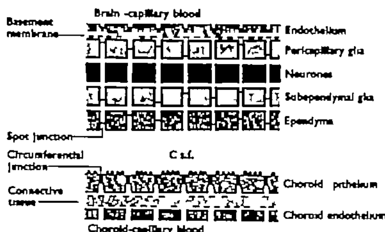


Fig. 27 Schematic representation of the structures involved in the exchange of materials between the blood, csf and cells within the vertebrate brain. The intercellular spaces between endothelial cells of "brain" capillaries are occluded by circumferential junctions (*zonulae occludentes*) which prevent the escape of small proteins, su h as horseradish peroxidase, from the blood. They may also prevent or retard the escape of smaller molecules. Circumferential junctions between choroid epithelial cells also act as a physical barrier for the escape of materials from the blood into the csf. However those molecules which do escape from "brain capillaries or from the choroid plexus have direct access to the neurones by diffusion via the fluid-filled intercellular spaces. The same is true even for molecules of 100 Å diameter like ferritin, when they are injected into the csf The spot junctions (*maculae occludentes*) between glia or ependyma do not impede diffusion through the clefts (From Cohen, Gerschenfeld and Kuffler 1968).

Fencl, Miller and Pappenheimer 1966), which was interpreted as indicating no net of HCO_3^- between brain extracellular fluid and blood, but the interpretation is erronous if the brain produces H^+ which titrate HCO_3^- In this situation there may be a net flux of HCO_3^- between blood and brain interstitial fluid across the capillaries but zero net flux between ventriculo-cisternal perfusion fluid and surrounding tissue, because the H^+ source (brain cells) is interposed between the venticular system and the capillaries in brain parenchyma.

The anatomical location of active transport of H^+ and/or HCO_3^- between blood and brain extracellular fluid. If the electrochemical potential difference for H^+ and HCO_3^- is maintained partly by a transcellular active transport of H^+ and/or HCO_3^- between blood and brain extracellular fluid such an active transport is most likely located in the cell layers which provide the major resistance to diffusion of the ions, i.e. the blood-brain barrier

Brightmann and Reese (1969) demonstrated intercellular circumferential tight junctions between the capillary endothelial cells in most areas of the brain and between the choroid plexus cells by their resistance to diffusion of tracers like horseradish peroxidase and it is probable that they also provide the major resistance to diffusion of small ions Cohen Gerschenfeld and Kuffler 1968) (Fig. 27). These cell layers are therefore probably the blood brain and the blood-csf barriers and the capillary endothelium and or the choroid plexus epithelium are therefore the probable

sites for transcellular transport of solutes between blood and brain extracellular fluid.

Because the choroid plexus cells transport other solutes (Davson 1967) they are a priori the most likely site for an active transport of H^+ and/or HCO_3^- between blood and brain extracellular fluid, but other evidence indicates that the electrochemical potential difference for HCO_3^- between csf and blood may be due to processes occurring at locations other than the choroid plexus.

Ames, Sakanoue and Endo (1964) compared the chloride concentrations in plasma ultrafiltrate, choroid plexus fluid and cisternal fluid and found that the concentrations were nearly the same in plasma ultrafiltrate and choroid plexus fluid whereas the concentration in cisternal fluid was higher indicating that the bicarbonate concentration decreases during the passage of the fluid through the venticular system. The lack of an effect of acetazolamide on the chloride concentration in choroid plexus fluid (Ames, Higashi and Nesbett 1965) may also be taken as indirect evidence against an active transport of H^+ and HCO_3^- across the choroid plexus epithelium.

Concluding remarks about the factors which determine the steady state distribution of H^+ and HCO_3^- between csf and blood During normoxia with a normal blood acid base composition H^+ and HCO_3^- are not in electrochemical equilibrium across the blood-brain and blood-csf barriers.

The electrochemical disequilibrium is probably due to an active transport system pumping either H^+ from blood to csf or HCO_3^- in the opposite direction During chronic blood acid base changes and during chronic hypoxia the electrochemical potential differences change. These changes may be explained partly by interference with an active transport of H^+ of HCO_3^- between blood and csf and partly by changes in metabolic H^+ production in the brain.

These mechanisms determine the steady state distribution of H^+ and HCO_3^- between blood and csf or brain extracellular fluid but it must be emphasized that the relative stability of the brain extracellular fluid pH during chronic hypoxia and chronic blood acid-base changes is partially attributable to the effect of pH changes in brain extracellular fluid on ventilation.

Chapter 7

Concluding remarks about the chemical control of ventilation during chronic blood acid base changes and during chronic hypoxia

In the previous chapter we described mechanisms which might affect the distributio of H^+ and HCO_3^- between csf and blood but we shall now examine how thes mechanisms interfere with the chemical control of ventilation during chronic bloo acid-base changes and during chronic hypoxia.

Metabolic acidosis When blood pH is suddenly lowered by infusing non-volatil acid, ventilation increases and csf pH increases as described in Chapter 3 If $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO^-}$ remained constant the changes in the steady state distribution of H^+ and HCO_3^- between csf and blood would be determined only by the change in the electrical potential difference csf blood. A steady state would be attained by a decrease in csf pH and by an increase in arterial pH the relative pH changes in the two compartments being determined by the ventilatory response to pH changes in arterial blood and in brain extracellular fluid. However during metabolic acidosis $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO^-}$ decrease and in steady state during metabolic acidosis the csf pH will be higher than if $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO^-}$ had remained constant. During metabolic acidosis *the effect of the changes in active transport of H^+/HCO_3^- between brain and* is therefore to preserve the pH in brain extracellular fluid on the "expense" arterial pH.

Metabolic alkalosis During metabolic alkalosis the pH changes are opposite in di rection but the changes in active transport of H^+/HCO_3^- between brain and blood also preserve pH in brain extracellular fluid on the "expense" of arterial pH.

Respiratory acidosis If ventilation is suddenly decreased for instance by increasing the resistance to breathing, P_{CO_2} increases and pH in blood and csf decreases. If $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO^-}$ remained constant the changes in the steady state distribution of H^+ and HCO_3^- between csf and blood would be determined only by the change in the electrical potential difference csf blood. A steady state would be attained by a decrease in csf pH and an increase in blood pH the relative pH changes in the two compartments being determined by the ventilatory response to pH changes in arterial blood and in brain extracellular fluid. However during respiratory acidosis $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO^-}$ increase, and in the steady state the pH in csf will be lower than if $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO^-}$ had remained constant. During this condition the change in active transport of H^+/HCO_3^- between brain and blood is therefore not "useful" in terms of maintaining pH stability in brain extracellular fluid.

The "usefulness" of those mechanisms is however appreciated when examining the events if $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO^-}$ remained constant during chronic respiratory acidosis. The initial steady state distribution would be attained by an increase in blood pH

and a decrease in csf pH. As bicarbonate reabsorption by the kidney increases blood pH increases causing a fall in the electrical potential difference csf blood and csf pH would return towards normal. The chemical drive to ventilation would therefore decrease causing a further decrease in ventilation and increase in P_{CO_2}. Therefore if $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$ remained constant the increase in P_{CO_2} during respiratory acidosis would be limited only by the kidney's ability to increase bicarbonate reabsorption.

However $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$ increase during respiratory acidosis and when blood pH returns towards normal csf pH therefore remains lower than normal. The increase in $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$ during respiratory acidosis therefore provides a feed back mechanism which limits the increase in P_{CO_2} when the work of breathing increases. The changes in $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$ during respiratory acidosis due to respiratory failure may be caused both by the effect of respiratory acidosis per se and by an effect of concommitant hypoxia.

Respiratory alkalosis During respiratory alkalosis the pH changes are opposite in direction. Like during respiratory acidosis the mechanisms discussed in Chapter 6 can not be considered "useful" in terms of maintaining a stable pH in the brain. However the described mechanisms are "useful" in that they provide a feed back mechanism which limits the changes in ventilation and P_{CO_2} when an additional respiratory stimulus is sustained.

Chronic hypoxia When P_{O_2} in inspired air is lowered ventilation increases even if it does not increase immediately via peripheral chemoreceptor stimulation. In the absence of peripheral chemoreceptor stimulation the increase in ventilation may be explained by the effect of hypoxia per se on $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$ (see Chapter 6). The ensuing hypocapnia creates a respiratory alkalosis in blood, which will tend to lower $\Delta\mu_{H}$ and $\Delta\mu_{HCO}$ hereby providing a feed-back mechanism which counteracts the effect of hypoxia on $\Delta\mu_{H}$ and $\Delta\mu_{HCO}$.

When ventilation is increased acutely via hypoxic stimulation of the peripheral chemoreceptors the pH increases in blood and csf. If $\Delta\mu_{H^+}$ and $\Delta\mu_{HCO}$ remained constant the steady state distribution of H and HCO_3^- between blood and csf would be determined only by the change in the electrical potential difference csf blood. However during respiratory alkalosis $\Delta\mu_{H}$ and $\Delta\mu_{HCO}$ decrease providing a feed-back mechanism which limits the effect of peripheral chemoreceptor stimulation on ventilation and P_{CO_2}. The importance of such a feed-back mechanism is appreciated when considering that if $\Delta\mu_{H}$ and $\Delta\mu_{HCO^-}$ remained constant then the increase in ventilation and the fall in P_{CO_2} for a certain peripheral chemoreceptor stimulation during chronic hypoxia would be limited only by the kidney's ability to decrease bicarbonate reabsorption.

Conclusion We must conclude that the mechanisms which are discussed in Chapter 6 provide feed-back mechanisms which must be considered essential for the attainment of a new steady state during chronic blood acid base changes and during chronic hypoxia.

hypoxia, whereas the high altitude natives who have been residing at high altitude only during childhood show an irreversibly blunted response to acute hypoxia. Because children born with cyanotic heart disease also show a low ventilatory response to acute hypoxia it is concluded that the blunted response in high altitude natives is not genetically determined but caused by chronic hypoxia during childhood. The anatomical location of the "lesion" which is responsible for the low ventilatory response to hypoxia has not been defined. It has not been possible to find an animal which exhibits the same phenomenon. Attempts to define the anatomical lesion in man from measurements of the sensitivity of the peripheral chemoreceptors to CO_2 are described.

Described in Chapter 5 are the ventilatory changes during chronic hypoxia. Ample evidence indicates that a lowering of brain extracellular fluid $[HCO_3^-]$ during chronic hypoxia is instrumental in the ventilatory changes during chronic hypoxia. Severinghaus et al. (1963) proposed that the changes in cerebrospinal fluid $[HCO_3]$ were due to changes in the rate of active transport of H or HCO_3 between blood and csf. According to this proposal the changes in brain extracellular fluid $[HCO_3]$ were initiated by the peripheral chemoreceptor induced hyperventilation. In essence they proposed that the changes in brain extracellular fluid $[HCO_3^-]$ were only secondary to the hypocapnia resulting from hypoxic stimulation of the peripheral chemoreceptors and no changes should occur if hypoxia did not elicit hyperventilation via the peripheral chemoreceptors. Evidence is presented that man and animals indeed hyperventilate during chronic hypoxia even if ventilation is not increased acutely when they are exposed to hypoxia. It is proposed that an increased anaerobic glycolysis in the brain is the primary event, which initiates and maintains a lowering of cerebrospinal fluid HCO_3^- during chronic hypoxia at least in the absence of peripheral chemoreceptor mediated hyperventilation.

In order to explain which factors affect cerebrospinal fluid $[HCO_3^-]$ during chronic hypoxia the effect of changes in blood acid base composition (respiratory alkalosis) must however also be taken into account. Chapter 6 describes the effects of blood acid-base changes and hypoxia on the distribution of H and HCO_3 between blood and csf. The ions are not in electrochemical equilibrium across the blood-brain barrier. The electrochemical disequilibrium may be explained by 1) an active transport of H and/or HCO_3^- between blood and csf and/or 2) a metabolic brain H source. During chronic hypoxia and chronic blood acid-base disorders, alterations in the electrochemical potential differences for the ions between blood and csf may be explained by interference with these two mechanisms. The role of various factors in determining the magnitude of the electrochemical potential differences for H and HCO_3 are discussed.

The role of the mechanisms discussed in Chapter 6 are discussed in Chapter 7 in terms of their interaction with the ventilatory control system i.e. the peripheral chemoreceptors and the central chemosensitive areas during chronic hypoxia and chronic blood acid base changes. It is concluded that the effects of blood acid-base

Summary

In Chapter 1 the structure and the function of the peripheral chemoreceptors are d scribed They the carotid and aortic bodies, are stimulated by a lowering of arter P_{O_2} or pH or by an increase in arterial P_{CO_2}. The stimulus-response curves for all th stimuli have been fairly well defined for the carotid bodies in the cat, but little known about other species. Little is known about how the mentioned stimuli transformed to an afferent nerve impulse from the peripheral chemoreceptors. cause the "mean" tissue P_{O_2} in the carotid bodies probably is close to arterial l the response elicited by small changes in arterial P_{O_2} even around an arterial P_{O_2} 100 mm Hg suggests that the carotid bodies show some change in metabolism w "mean" tissue P_{O_2} changes around this level. Recent evidence indicates that carotid bodies are innervated by efferent fibers, but the role of this innervation carotid body stimulation remains unknown

In Chapter 2 the central chemosensitive areas are described. These areas wl respond to pH changes in brain extracellular fluid are located in the brain but t exact location remain unknown. It is not known whether there are specific "t receptors in the brain stem or whether ventilatory responses to pH changes in b extracellular fluid are elicited via the respiratory neurons in general. The probl ed in defining the stimulus-response characteristics of the central chemosensi are discussed.

Chapter 1 and 2 the peripheral chemoreceptors and the central chemosens areas are described in terms of their individual stimulus-response characteristics Chapter 3 the interaction between the different areas is discussed, in order to scribe the events during an acute change in arterial P_{O_2} P_{CO_2}, or pH The interac between peripheral and central chemosensitive areas causes problems in designin test which measures the sensitivity of one of the receptor sites to any one of particular stimuli. The problems encountered in attempts to measure the sensitiv of the peripheral chemoreceptors to a lowering of P_{O_2} and an increase in P_{CO_2} discussed. *However all test which have been used to measure the ventilatory resp* to acute hypoxia, despite theoretical objections, distinguish between the respon normal sea level natives and the blunted response observed in people who are l and who have lived at high altitude

Chapter 4 describes evidence that man who is born at high altitude (high alti natives) show a blunted ventilatory response to acute hypoxia. Evidence is prese that man who is born at sea level but who has been residing at high altitude for r years during adult life does not develop a blunted ventilatory response to t

Summary in Danish

I kapitel 1 beskrives de perifere kemoreceptorer carotislegemerne og aortalegemerne Disse kan stimuleres ved en nedsættelse af arterielt Po_2 eller pH eller ved en øgning af arterielt Pco_2 For disse stimuli foreligger en række undersøgelser over stimulus-response karakteristikken for carotislegemerne hos katte hvorimod der kun er sparsomme oplysninger om andre dyrearter Det vides ikke hvorledes disse stimuli transformeres til afferente nerveimpulser fra kemoreceptorerne. I carotislegemerne er den gennemsnitlige vævstension for ilt sandsynligvis meget nær ved den arterielle tension, og det forhold at selv små ændringer i arterielt Po_2 omkring et Po_2 på 100 mm Hg påvirker impulsudsendelsen fra carotislegemerne, tyder på at stofskiftet i carotislegemerne påvirkes ved ændringer i vævs Po_2 omkring dette niveau Nylige undersøgelser har vist, at carotislegemerne modtager efferent innervation, men det vides ikke, om denne innervation er af betydning for virkningen af hypoxi eller acidose på carotislegemerne.

I kapitel 2 beskrives de centrale kemosensitive områder Disse områder stimuleres af en nedsættelse af pH i hjernes extracellulære væske. Den nøjagtige anatomiske lokalisation af de centrale kemosensitive områder kendes ikke og det vides heller ikke, om virkningen af pH ændringer skyldes en virkning på specifikke pH receptorer i hjernestammen eller om det er en virkning på de respiratoriske neuroner som helhed. I dette kapitel diskuteres også de problemer man møder ved forsøg på at definere stimulus-response karakteristikken for de centrale kemosensitive områder

I kapitel 1 og 2 er de perifere kemoreceptorer og de centrale kemosensitive områder forsøgt beskrevet med henblik på stimulus-response karakteristikken for de enkelte områder I kapitel 3 beskrives interaktionen mellem de to områder med henblik på en beskrivelse af de ventilatoriske ændringer ved akutte ændringer i arterielt Po_2, Pco_2 og pH Interaktionen mellem perifere kemoreceptorer og centrale kemosensitive områder skaber problemer når man skal opstille en metode, der kan bruges til at undersøge følsomheden af det ene af »receptor«-områderne for et af de nævnte stimuli dette demonstreres ved de teoretiske problemer der foreligger ved valget af metoder der måler følsomheden af de perifere kemoreceptorer for hypoxi og hypercapni. Det fremhæves imidlertid at alle de metoder der har været anvendt til at måle følsomheden af de perifere kemoreceptorer for hypoxi har været i stand til at skelne mellem følsomheden hos mennesker der er født og opvokset i lavlandet (sea level natives) og den nedsatte følsomhed hos mennesker der er født og opvokset i bjergegne (high altitude natives) trods teoretiske begrænsninger af de omtalte metoder

I kapitel 4 diskuteres de experimentelle fund der har vist, at high altitude natives udviser et nedsat ventilatorisk response til akut hypoxi sammenlignet med det venti-

changes on the electrochemical potential differences for H^+ and HCO_3^- between b
and csf provides an essential feedback mechanism, which limits the changes in }
(ventilation) during respiratory acidosis and alkalosis. The mechanisms may how-
also be considered advantageous during metabolic acidosis and alkalosis and du
chronic hypoxia.

Summary in Danish

I kapitel 1 beskrives de perifere kemoreceptorer carotislegemerne og aortalegemerne. Disse kan stimuleres ved en nedsættelse af arteriel P_{O_2} eller pH eller ved en øgning af arterielt P_{CO_2}. For disse stimuli foreligger en række undersøgelser over stimulus-response karakteristikken for carotislegemerne hos katte hvorimod der kun er sparsomme oplysninger om andre dyrearter. Det vides ikke hvorledes disse stimuli transformeres til afferente nerveimpulser fra kemoreceptorerne. I carotislegemerne er den gennemsnitlige vævstension for ilt sandsynligvis noget nær ved den arterielle tension, og det forhold at selv små ændringer i arterielt P_{O_2} omkring et P_{O_2} på 100 mm Hg påvirker impulsudsendelsen fra carotislegemerne tyder på at stofskiftet i carotislegemerne påvirkes ved ændringer i vævs P_{O_2} omkring dette niveau. Nyligе undersøgelser har vist, at carotislegemerne modtager efferent innervation men det vides ikke om denne innervation er af betydning for virkningen af hypoxi eller acidose på carotislegemerne.

I kapitel 2 beskrives de centrale kemosensitive områder. Disse områder stimuleres af en nedsættelse af pH i hjernes extracellulære væske. Den nøjagtige anatomiske lokalisation af de centrale kemosensitive områder kendes ikke og det vides heller ikke, om virkningen af pH ændringer skyldes en virkning på specifikke pH receptorer i hjernestammen eller om det er en virkning på de respiratoriske neuroner som helhed. I dette kapitel diskuteres også de problemer man møder ved forsøg på at definere stimulus-response karakteristikken for de centrale kemosensitive områder.

I kapitel 1 og 2 er de perifere kemoreceptorer og de centrale kemosensitive områder forsøgt beskrevet med henblik på stimulus-response karakteristikken for de enkelte områder. I kapitel 3 beskrives interaktionen mellem de to områder med henblik på en beskrivelse af de ventilatoriske ændringer ved akutte ændringer i arterielt P_{O_2}, P_{CO_2} og pH. Interaktionen mellem perifere kemoreceptorer og centrale kemosensitive områder skaber problemer når man skal opstille en metode, der kan bruges til at undersøge følsomheden af det ene af »receptor«-områderne for et af de nævnte stimuli dette demonstreres ved de teoretiske problemer der foreligger ved valget af metoder der måler følsomheden af de perifere kemoreceptorer for hypoxi og hypercapni. Det fremhæves imidlertid at alle de metoder der har været anvendt til at måle følsomheden af de perifere kemoreceptorer for hypoxi, har været i stand til at skelne mellem følsomheden hos mennesker der er født og opvokset i lavlandet (sea level natives) og den nedsatte følsomhed hos mennesker der er født og opvokset i bjergegne (high altitude natives), trods teoretiske begrænsninger af de omtalte metoder.

I kapitel 4 diskuteres de experimentelle fund, der har vist, at high altitude natives udviser et nedsat ventilatorisk response til akut hypoxi sammenlignet

latoriske response til akut hypoxi sammenlignet med det ventilatoriske response hos sea level natives. Det vises yderligere, at det ventilatoriske response til akut hypoxi ikke nedsættes hos sea level natives selv efter et mangeårigt ophold i bjergene, samt at det lave ventilatoriske response hos high altitude natives er irreversibelt selv efter et mangeårigt ophold i lavlandet. Det synes udelukket at genetiske faktorer er af væsentlig betydning for disse forskelle, fordi mennesker der har haft en hjertelidelse med cyanose i barnealderen (Steno-Fallots tetralogi) også udviser et lavt ventilatorisk response til akut hypoxi Det vides ikke, hvor denne forskel er lokaliseret anatomisk, blandt andet fordi forsøg på at finde dyreart, der udviser dette fænomen har været negative Forsøg på at skelne mellem en perifer og en central lokalisation af dette »fænomen« på grundlag af målinger af den perifere kemoreceptor følsomhed for CO_2 hos mennesker omtales.

I kapitel 5 beskrives de ventilatoriske ændringer ved kronisk hypoxi Det er veldokumenteret, at en nedsættelse af $[HCO_3^-]$ i hjernens extracellulære væske er af væsentlig betydning for ændringerne i ventilationen ved kronisk hypoxi. Severinghaus et al. (1963) fremsatte en hypothese, der forklarede ændringerne i $[HCO_3^-]$ i hjernens extracellulære væske ved en ændring i hastigheden af en aktiv transport af H^+ eller HCO_3^- mellem blod og cerebrospinal væske Ifølge denne hypothese var faldet i HCO_3^- i hjernens extracellulære væske ved kronisk hypoxi udløst af ventilationsstigningen og hypocapnien forårsaget af stimulation af de perifere kemoreceptorer som følge heraf skulle dyr eller mennesker ikke hyperventilere ved kronisk hypoxi, hvis ventilationen ikke øgedes via stimulation af de perifere kemoreceptorer Det fremføres, imidlertid, at både dyr og mennesker hyperventilerer ved kronisk hypoxi, om der ikke er nogen akut ventilationsstigning udløst via de perifere kemo- ... Det foreslås derfor at en øget anaerob glykolyse i hjernen er den primære ...r der udløser og vedligeholder en nedsættelse af $[HCO_3^-]$ i hjernens extracellulære væske ved kronisk hypoxi i hvert fald når hypoxi ikke udløser hyperventilation via stimulation af de perifere kemoreceptorer

En beskrivelse af de faktorer der øver indflydelse på $[HCO_3^-]$ i hjernens extracellulære væske ved kronisk hypoxi må inkludere en beskrivelse af virkningen af ændringer i blodets syre-base komposition (respiratorisk alkalose) I kapitel 6 skrives virkningen af ændringer i blodets syre-base komposition og af kronisk hy... på fordelingen af H^+ og HCO_3^- mellem blod og cerebrospinalvæske. Disse j... ikke i elektrokemisk ækvilibrium mellem blod og cerebrospinalvæske hvilk... forklares ved 1) en aktiv transport af H^+ og/eller HCO_3^- mellem blod og ... væske og/eller 2) en metabolisk H^+ kilde i hjernen. Ændringer i de potential differenser for disse ioner ved kronisk hypoxi og ved kroniske blodets syre-base komposition kan forklares ved en virkning af disse to ... Virkningen af forskellige faktorer på de elektrokemiske potentialforskelle ... HCO_3^- diskuteres.

I kapitel 7 diskuteres, hvorledes de mekanismer der er omtalt i ... betydning for den intakte organisme, når de betragtes med henblik på ... med den kemiske kontrol af ventilationen d.v.s med de perifere

de centrale kemosensitive områder. Det konkluderes, at virkningen af ændringer i blodets syre-base status på de elektrokemiske potentialdifferenser for H og HCO_3^- mellem blod og cerebrospinalvæske er en essentiel feed back mekanisme, der begrænser ændringerne i P_{CO_2} ved respiratorisk acidose og alkalose. Disse mekanismer må imidlertid også betragtes som hensigtsmæssige ved metabolisk acidose og ved kronisk hypoxi.

References

The numbers in brackets indicat the pages and figures on which the reference

ÅSTRAND, P O A study of chemoreceptor activity in animals exposed to prolong *Acta physiol Scand.* *1954* *30* 335–342. (33).

ALEXANDER, S. C., P J COHEN H. WOLLMAN, T C. SMITH, and M REIVICH. Cerebr drat metabolism during hypocarbia in man. *Anesthesiology* 1965 *26.* 624–632

ALEXANDER, S. C., T C SMITH, G STROBEL, G W STEPHEN, and H. WOLLMAN. Cerel hydrate metabolism of man during respiratory and metabolic alkalosis. *J appl Ph* *24* 66–72. (52).

AMES, A., M SAKANOUE, and S. ENDO. Na K, Ca, Mg and Cl concentrations in choro fluid and cisternal fluid compared with plasma ultrafiltrate. *J Neurophysiol* 1964 *27* (55).

AMES, A. K. HIGASHI and F B. NESBETT Effects of P_{CO_2}, acetazolamide nd ouabain on and composition of choroid plexus fluid. *J Physiol (Lond.).* 1965 *181* 516–524 (

BAIN, J A. and J R. KLEIN. Effect of carbon dioxide on brain glucose lactate, pyru phosphates. *Amer J Physiol* 1949 *158* 478–484 (52)

BISCOE, T J nd W E. STEHBENS. Ultrastruct re of the carotid body *J Cell Biol* 1966. *30* 56 (14).

BISCOE, T J and W E STEHBENS. Ultrastructure of the denervated carotid body *Quart J Physiol* 1967 *52* 31–36. (14)

BISCOE, T J and M J PURVES. Factors affecting the cat carotid chemoreceptor nd cer sympathetic activity with special reference to passive hind-limb movements. *J Physiol. (Lo* 1967 *190* 425–441 (12).

T J S. R. SAMPSON and M J PURVES. Stimulus response curves of single carotid b eepto afferent fibers. *Nature (Lond).* 1967 *215* 654–655 (12, 30).

, T J nd S R. SAMPSON. Spontaneous activity recorded from the central cut end of t cá otid sinus nerve of the cat. *Nature (Lond.).* 1967 *216* 294–295. (11).

BJURSTEDT A. G H. Interaction of centrogenic and chemoreflex control of breathing duri oxygen deficiency at rest. *Acta physiol. Scand.* 1946. *1.*, suppl. 38. (33).

BLACK, A. M S., D I MCCLOSKEY and R. W TORRANCE. The responses of peripheral chemore ceptors to sudden changes of hypercapnic and hypoxic stim li. *J Physiol. (Lond.).* 1966 *185* 67 P (13).

BLEICH H L., P M BERKMAN, and W B. SCHWARTZ. The response of position t sustained hypercapnia. *J In. Invest* 1964 *43* 11–16. (52, fig.

BOUVEROT P., R. FLANDROIS, R. PUCCINELLI nd P DEJOURS. Étude d role des artérioles dans la régulation de la respiration dans le chien éveillé. *Arch Int* 1965 *157* 253– 71 (29 31).

BRADBURY M W B. nd C R. KLEEMAN Stability f the potassium fluid and brain. *Amer J Ph siol* 1967 *213* 519–528 (43).

BRADLEY R. D and S. J G SEMPLE. A comparison of certain cid base ch blood, jugular venous blood and cerebrospinal fluid in man, and the acute and chronic acid-base disturbances. *J Physiol. (Lond.).* 1962. 2., 23).

BRIGHTMAN, M. W nd T S REESE. J actions between intimately appo the ertebrat brain. *J cell Biol* 1969 *40* 648–677 (54).

BOWES, C. Mc C., F F KAO, and B B LLOYD (ed.) Cerebrospin l fluid nd the regul tion of respiration. *Oxford, Black ll Scientific Publ* 1965 (10)
BOWES, J. G and S M TENNEY Ventilatory response of llama to hypoxia at sea lev l and h gh altitude *Resp Physiol.* 1968 5 69 78 (79).
BROWN, E. B G S. CAMPBELL, M N JOHNSON, A. HEMINGWAY and M B VISSCHER Ch nges in response to inhalation of CO_2 before and after 4 hours of hyperventilation in man *J appl Physiol* 1948 1 333–338. (35)
BROWN, E. B G S. CAMPBELL J O ELAM F GOLLAN, A. HEMI GWAY and M B VISSCHER Electrolyte changes with chronic pa i e hyperventilation in man *J appl Ph siol* 1949 1 848-855 (35).
BROWN E. B A. HEMINGWAY and M B VISSCHER Arterial blood pH and P_{CO_2} ch nges in re sponse to CO_2 inhalation after 4 hours of p si e hyperve tilation. *J appl Ph iol* 1950 – 544-548. (35).
BROWN, E. B. Cha ges in brain pH response to CO_2 fter prolonged hypervent lation. *J appl Physiol.* 1950 – 549 55– (35).
C MERON, I. R. Discussion. In *Alfred Benzon S mposi m III Ion Homeo tasis of the Brain* Ed. by B. K. Siesjö and S. C. Sorensen. Munk gaa d, Copenhagen 1971 119 1 1 (43).
CERRETELLI, P Esistenza di una permanent stimolazione ipossica del centro respiratorio in individui acclimatati a quote di 5000–7500 M.s.l m. (Himalay) Atti del Congresso Inter nazionale di Medicina. *Aeronautica e Spaziale* 1961 2 866–873 (33).
CHIODI, H. Respiratory adaptations to chronic high ltitude hypoxia *J appl Physiol* 1957 10 81–87 (20, 26).
CHIODI, H. Respiratory adaptation to high altitude In *Th egulation of human respiration.* Ed by D J C. Cunningham and B. B Ll yd, Oxford, Blackwell Scientific P bl., 1963 336–378. (26).
COHEN, M M The central nervous ystem in congenital heart dise se *Neurology (Minneap)* 1960. 10 45 –456. (32).
COHEN, M. W H. M GERSCHENFELD nd S W KUFFLER. Ionic environment of Neurones nd Glial Cells in the brain of an Amph bia *J Ph siol (Lond).* 1968 197 363–380 (43 54)
COHEN, P J S. C. ALEXANDER T C SMITH, M RE ICH, and H WOLLMAN. Effect f hypoxia and normocarbia on cerebral blood flow nd metabolism in conscious man. *J appl Physiol* 1967 23 183–189 (39 40 51 53)
COMROE, J H., JR. The location nd functio of the chemoreceptors f the orta. *Amer J Physiol* 1939 127 176–191 (14).
COMROE, J H., JR. The peripheral hemoreceptors. I *Handbook of Physiology Respiration* vol. 1 Washington, D C Amer Physiol Soc. 1964. pp 557 583. (9 10, 11 14).
COZINE, R. A. and S H. NGAI. Med llary surface chemoreceptors and regulation of respiration in the cat. *J appl. Physiol* 1967 22. 117 121 (15).
CSERR, H. Potassium exchange between cerebrospinal fluid, plasma and brain. *Amer J Ph siol* 1965 209 1219–1226 (43).
CUNNINGHAM, D J C D G SH w S. LAHIRI nd B. B. LLOYD. The effect f maintained ammo nium hloride acidosis on the relation bet een pulmonary ventilation nd lveolar oxygen and carbon dioxide in ma *Quart J p. Physiol.* 1961 46, 323–334 (19).
CUNNINGHAM, D J C E. N HEY J M P TRICK, and B. B. LLOYD. The effect of noradrenaline infusion on the relatio between pulmonary ventilatio and the alveolar P_{O_2} and P_{CO_2} in man. *Ann. N Y A ad Sci* 1963 109 756–771 (12).
CUNNINGHAM, D J C d B. B. LLOYD. (ed.) *The regulation of huma espiration.* Oxford, Black well Scientific Publ, 1963 (10).
D LY M. DE B., C. J LAMBERTSEN, and A. SCHWEITZER. Observations on the volume of blood flow and oxygen utilisation of the carotid body in the cat *J Physiol (Lond.)* 1954 125 67–89 (12, 13).

References

The numbers i brackets indicate the pages and figures on which the references is cited

ASTRAND P O A study of chemoreceptor activity in animals exposed to prolonged hypoxia. *Acta physiol. Scand.* 1954 30. 335–342. (33)

ALEXANDER, S. C., P J COHEN, H WOLLMAN, T C. SMITH, and M REIVICH. Cerebral carbohydrate metabolism during hypocarbia in man. *Anesthesiology* 1965. 26 624–632. (52).

ALEXANDER, S. C., T C. SMITH, G STROBEL, G W STEPHEN, and H. WOLLMAN. Cerebral carbohydrat metabolism of man during respirat ry and metabolic alkalosis. *J ppl Physiol.* 1968. 24 66–72. (52).

AMES, A., M. SAKANOUE, and S. ENDO. Na, K, Ca Mg and Cl concentrations in choroid plexus fluid nd cisternal fluid compared with plasma ultrafiltrate *J Neurophysiol* 1964 27 672–681 (55).

AMES, A., K. HIGASHI a d F B. NESBETT Effects f P_{CO_2}, acetazolamide and ouabai on volume d composition f choroid plexus fluid *J Physiol (Lond).* 1965 *181* 516–524 (55)

BAIN, J A and J R. KLEIN. Effect of carbon dioxide on brain glucose, lactate, pyruvate and phosphates. *Amer J Physiol* 1949 *158* 478–484 (52).

BISCOE, T J and W E. STEHBENS. Ultrastructure of the carotid body *J Cell Biol.* 1966. 30. 563–578. (14).

BISCOE, T J and W E. STEHBENS. Ultrastructure of the denervated carotid body *Quart J exp Physiol* 1967 52 31–36. (14)

BISCOE, T J and M J PURVES. Factors affecti g the cat carotid chemoreceptor and cervical sympathetic activity with special reference to passive hind-limb movements *J Physiol (Lond.)* 1967 *190* 425–441 (1?).

T J S R. SAMPSON and M J PURVES. Stimulus response curves of si gle carotid body fferent fibers. *N ture (Lond.)* 1967 *215* 654–655 (12, 10)

...e, T J and S. R. SAMPSON Spontaneous activity reco ded from th central cut end f the carotid sinus nerve of the cat. *Nature (Lond.).* 1967 *216* 294–295 (11).

BJURSTEDT A. G H Interaction of centrogenic and chemoreflex control of breathing during oxygen deficiency at rest. *Acta ph siol Scand.* 1946. *12* suppl. 38. (33).

BLACK, A. M S. D I MCCLOSKEY and R W TORRANCE. The responses of peripheral chemoreceptors to sudden cha ges of hypercap ic and hypoxic stimuli. *J Physiol. (Lond.)* 1966. *185* 67 P (13)

BLEICH, H. L., P M. BERKMAN and W B. SCHWARTZ. The response of cerebrospinal fluid composition to sustained hypercap ia. *J clin Invest* 1964 *43* 11 16. (52, fig 22, 23).

BOUVEROT P R. FLANDROIS, R. PUCINELLI, and P DEJOURS. Étude du role des chémorécepteurs artériels dans la régulation d l resp ration dans le chien éveillé. *Arch. int Pharmacodyn.* 1965 *157* 253– 71 (29 31).

BRADBURY M W B. nd C R. KLEEMAN Stability f the potassi m content of cerebrospinal fluid and brai *Amer J Ph siol* 1967 *213* 519–528. (43)

BRADLEY R. D and S J G. SEMPLE A comparison of certain cid base characteristics of arterial blood, jugular venous blood nd cerebrospinal fluid i man, and the effect on them of some acut and chronic cid-base dist rbances. *J Physiol (Lond.).* 196.. *160* 381 391 (fig. 21 22, 23)

BRIGHTMAN, M W and T S REESE. J nctions between intimately apposed cell membranes in the vertebrate brain. *J cell Biol* 1969 *40* 648–677 (54).

...C. Mc C. T. F. Kao, and B. B. Lloyd. (ed.) Cerebrospin... ...worton. Oxford, Black... ...Scientif. Publ. 1963. (10).

... J. G. and S. M. Tenney. Ventilatory response of llama to hyp... ...Resp. Physiol. 1968. 5: 69-78. (79).

... E. B., G. S. Campbell, M. N. Johnson, A. Hemingway, and M. ... ...ponse to inhalation of CO_2 before and after 24 hours of hype... Physiol. 1945. 1: 333-338. (35).

... E. B., G. S. Campbell, J. O. ... T. Gollan, A. Hemi... Electrolyte changes ...th chronic pa...ive hyperventilation in m... ...(35).

... E. B., A. Hemingway, and M. B. Visscher. Arterial blood ... ...ponse to CO_2 inhalation after 24 hours of pa...ive hypervent... ...(35).

... E. B. Changes in brain pH response to CO_2 ... Physiol. 1950. ...549-55... (35).

..., J. R. Discussion. In: Alfred Benz... Symposium... ...by B. K. Siesjö and S. C. Sørensen. Munksgaard. Copenh...

..., P. Esistenza di una permanente stimulazione impo... ...acclimatati a quote di 5000-7500 M s.l.m. ... ...Medicina Aeronautica e Spaziale. 1961. ...

..., B. Respiratory adaptations to chronic high altitude hypo... ...(25, 26).

..., B. Respiratory adaptation to high altitude. In: The reg... ...by D. J. C. Cunningham and B. B. Lloyd. Oxford, Blackwell S... ...(25).

... M. M. The central nervous system in congenital heart disease. ... 1960 ...: 452-455. (1).

... M. W., H. M. Gerschenfeld, and S. W. Kuffler. ...Glial Cells in the brain of an Amphibian. J. Physiol. (Lond.) 196... ...30 (43, 54).

..., P. J., S. C. Alexander, T. C. Smith, M. Reivich, and H. W. ... ...and normocarbia on cerebral blood flow and metabolism in ... J. Appl. Physiol. 1967. 23: 183-189. (39, 40, 51, 53).

..., J. H., Jr. The location and function of the chemoreceptors of the ... Amer. J. Physiol. 1939. 127: 176-191. (14).

..., J. H., Jr. The peripheral chemoreceptors. In: Handbook of Physiology. Respiration ...Vol. I. Washington, D. C., Amer. Physiol. Soc. 1964. pp. 557-583. (9, 10, 11, 14).

..., R. A., and S. H. Noat. Medullary surface chemoreceptors and regulation of respiration in the cat. J. Appl. Physiol. 1967. 22: 117-121. (15).

..., H. Potassium exchange between cerebrospinal fluid, plasma and brain. Amer. J. Physiol. 1965. 209: 1219-1226. (43).

Cunningham, D. J. C., D. G. Shaw, S. Lahiri, and B. B. Lloyd. The effect of maintained ammonium chloride acidosis on the relation between pulmonary ventilation and alveolar oxygen and carbon dioxide in man. Quart. J. Exp. Physiol. 1961. 46: 323-334. (19).

Cunningham, D. J. C., E. N. Hey, J. M. Patrick, and B. B. Lloyd. The effect of noradrenaline ... on the relation between pulmonary ventilation and the alveolar P_{O_2} and P_{CO_2} in man. Ann. N. Y. Acad. Sci. 1963. 109: 756-771. (12).

Cunningham, D. J. C., and B. B. Lloyd. (ed.) The regulation of human respiration. Oxford, Blackwell Scientific P..., 1963. (10).

De Castro, F. ... C. J. Lammertsen, and A. Schweitzer. Observations on the volume of blood ... ...ation of the carotid body in the cat. J. Physiol. (Lond.). 1954. 125: 67-89 (... 11).

DAVENPORT H W G BREWER, A H CHAMBERS, and S GOLDSCHMIDT The respiratory responses to anoxemia of unanesthetized dogs with chronically denervated aortic and carotid chemoreceptors and their causes. *Amer J Physiol* 1947 *148* 406–416. (36).

DAVSON H *General Physiology* London. J A. Churchill 1964 pp 1166. (42).

DAVSON, H *Physiology of cerebrospinal fluid* J A Churchill. London 1967 pp 445 (55).

DE CASTRO, F Sur la structure et l'innervation de la glande intercarotidienne (glomus caroticum) de l'homme et des mammifères et sur un nouveau systeme d innervation autonomme du nerf glossopharyngien Etudes anatomiques et experimentales. *Trabajo Lab I vest biol Uni Madrid* 1926. *24* 365–432. (9 11))

DECHARNAUX, G L influence de l altitude sur la respiration de deux chiens privés de leurs sinus carotidiens. *C R Soc biol (Paris)*. 1934 *116* 352 355 (36).

DEJOURS, P Y LABROUSSE, J RAYNAUD, and A TEILLAC Stimulus oxygene chemoreflexe de la ventilation a basse altitude (50 m) chez l Homme. *J Physiol (Paris)*. 1957 *49* 115–120. (20, 31).

DEJOURS P., Y LABROUSSE J RAYNAUD, and A. TEILLAC Etude de la régulation de la ventilation de repos chez l'homme en haute altitude. *Rev Franc Etud clin. biol* 1959 *4* 115–127 (33).

DRIPPS, R. D and J H COMROE, JR. The effect of the inhalation of high and low oxygen concentrations on respiration pulse rate ballistocardiogram and arterial oxygen saturation (oximeter) f normal individu l *Amer J Physiol* 1947 *149* 277 291 (20, 26, 37).

DUKE H N J H GREEN, and E. NEIL. Carotid chemoreceptor impulse activity d ring inhalation of CO_2 mixtures. *J Physiol (Lond)*. 1952. *118* 520–527 (14).

EGER E. I R H KELLOG A H MINES, M LIMA OTTOS, C. G MORRILL, and D W KENT Influence of CO_2 on ventilatory acclimatization to altitude. *J appl Physiol* 1968 *24* 607–615 (35)

ERBSLÖH F P KLÄRNER, and A BERNSMEIER. Die Milchsäureabgabe des menschlichen Gehirns. *Pflügers Arch ges Physiol* 1958 *268* 120–133 (53).

EYZAGUIRRE, C and J LEWIN. Chemoreceptor activity of the carotid body of the cat. *J Physiol (Lond.)* 1961a. *159* 222 37 (12)

EYZAGUI RE, C. nd J LEWIN. The effect of symphathetic stimulation on carotid nerve activity – *J Physiol (Lond)* 1961b. *159* 268–281 (12)

F S Oxygen consumption of the carotid body *Amer J Physiol* 1970. *218* 518–523 (13).

–, V T B MILLER and J R. PAPPENHEIMER. Studies on the respiratory response to disturbances of acid-base balance with deductions concerning the ionic composition of cerebral interstitial fluid. *Amer J Physiol* 1966 *210* 459–472. (23 45 54).

FENCL, V., J R. VALE, and J A. BROCH Respiration cerebral blood flow and composition of cerebral fluids during steady metabolic acidosis and alkalosis in humans. *J appl Physiol.* 1969 *27* 67 76. (23 fig. 21 22. 23).

FINN, H F F KAO, S. S. MEI and M H HARMEL. Csf blood potential in cats and its modification by sodium gammahydroxybutyrate *Arch int Pharmacodyn* 1968 *176* 319–325 (43).

FITZGERALD M P The changes in the breathing and the blood at various high altitudes. *Tra Roy Soc London, Ser B* 1913 *203* 351 371 (37).

FLOYD W F and E. NEIL. Infl ence of the sympathetic innervation of the carotid bifurcation on chemoreceptor and baroreceptor activity in the cat. *Arch. int Pharmacodyn* 1952. *91* 230– 40 (12)

GEMMILL, C. L. and D L. REEVES. Effect of anoxemia in normal dogs before and fter denervation of carotid chemoreceptors. *Amer J Physiol* 1933 *105* 487–495 (15 17 20, 36).

GERARD M W and P R. BILLINGSLEY The innervation of the carotid body *Anat Rec* 1923 *26* 391–400. (11).

GESELL, R and A. B. HERTZMA N. The regulation of respiration. IV Tissue acidity blood acidity and pulmonary ventilation. A study of the effects of semipermeability of membranes and the buffering action of tissues with the contin us method of recording changes in acidity *Amer J Physiol* 1926. *78* 610–6?9 (23).

GIBBS, E. L., W. G. LENNOX, L. F. NIMS, and F. A. GIBBS. Arterial and cerebral venous blood. Arterial-venous differences in man. *J. biol. Chem.* 194[illegible] *144*: 3[illegible]5–33[illegible]. (53)

GELFAND, R., J. T. HANSEN, R. H. KELLOGG, N. P. CHEN and E. M. CUTHBERTSON. Physiologic study on the chemoreceptor mechanism in the dog at sea level and at high altitude (12600 ft) (Abstract). *Circulation* 19[illegible] *18*: 724. (36).

GOTOH, C. Effect of chronic acidosis and alkalosis on rat csf-blood potential. *Physiologist* 1965. *8*: 178. (43 45)

GRAY, B. A. Response of the perfused carotid body to changes in pH and P_{CO_2}. *Resp. Physiol.* 1968 *4*: 229–45. (9 12, 14, 16)

GÖRMAN, N. and H. WINTERSTEIN. Die Wirkung von Hypoxie auf den pH von Hirnblut und Cerebrospinal-Flüssigkeit. *Pflüger Arch. ges. Physiol.* 1958 266: 318–323. (17).

HALL, P., Y. MATSUYAMA, K. R. WILLIAMS, R. GELFAND, and C. J. LAMBERTSEN. Effect of oxygen at 0.2, 1 and 3.5 atm. abs. on carotid chemoreceptor discharge in the cat at various levels of carbon dioxide. *Fed. Proc.* 1967 26: 379. (21 31).

HASSELBALCH, K. A., and J. LINDHARD. Analyse des Höhenklimas in seinen Wirkungen auf die Respiration. *Skand. Arch. Physiol.* 1911 *25*: 361–408. (33).

[illegible] HEIST, A. N. P., A. H. MAAS, and B. F. VISSER. L equilibre acido-basique dans le sang et le liquide cephalo-rachidien dans l'hypercapnie chronique. *Entret. Physio-Pathol. Respir.* Nancy 1964. (fig. 21, 22, 23).

HELD, D. R., V. FENCL, and J. R. PAPPENHEIMER. Electrical potential of cerebrospinal fluid. *J. Neurophysiol.* 1964 *27*: 942–959. (35 4[illegible], 43 45 46, 50).

HEYMANS, J. F. and C. HEYMANS. Sur les modifications directes et sur la régulation réflexe de l'activité du centre respiratoire de la tete isolée du chien. *Arch. int. Pharmacodyn.* 1927 *33*: 272–370. (9 11 14).

HEYMANS, C., J. J. BOUCKAERT and L. DAUTREBANDE. Sinus carotidien et reflexes respiratoires. II. Influences respiratoires reflexes de l acidose, de l'alcalose, de l'anhydride carbonique, de l'ion hydrogene et de l'anoxemie. Sinus carotidien et echanges respiratoires dans les poumons et au de dela des poumons. *Arch. int. Pharmacodyn.* 1930. *39*: 400–450. (11).

HEYMANS, C. and E. NEIL. *Reflexogenic areas of the cardiovascular system.* Boston, Little, Brown and Co. 1958. (11 14).

HORNBEIN, T. F., Z. J. GRIFFO, and A. ROOS. Quantitation of chemoreceptor activity. Interrelation of hypoxia and hypercapnia. *J. Neurophysiol.* 1961 *24*: 561–568. (9 12).

HORNBEIN, T. F. and A. ROOS. Specificity of H ion concentration as a carotid chemoreceptor stimulus. *J. appl. Physiol.* 1963 *18*: 580–584. (9 1[illegible] 14).

HORNBEIN, T. F. The relation between stimulus to chemoreceptors and their response. In *Arterial Chemoreceptors.* Ed. by R. W. Torrance, Oxford, Blackwell Scientific Publ. 1968. 65–78. (14 fig. 2).

HORNBEIN, T. F. and S. C. SORENSEN. Ventilatory response to hypoxia and hypercapnia in cats living at high altitude. *J. appl. Physiol.* 1969 *27*: 834–836. (29).

JOELS, N. and E. NEIL. The influence of anoxia and hypercapnia separately and in combination on chemoreceptor impulse discharge. *J. Physiol. (Lond.)*. 1960. *155*: 45–46. (12).

KATSAROS, B., H. H. LOESCHCKE, D. LERCHE, H. SCHÖNTHAL, and N. HAHN. Wirkung der bikarbonat-alkalose auf die Lungenbelüftung beim Menschen. Bestimmung der Teilwirkungen von pH und CO_2 – druck auf die Ventilation und vergleich mit dem Ergebnissen bei acidose. *Pflügers Arch. ges. Physiol.* 1960. *271*: 732–747. (15 21).

KATZ, B. *Nerve, muscle and synapse.* New York, McGraw-Hill, 1966. pp. 191. (42).

KELLOGG, R. H., N. P. CHEN, E. R. ARCHIBALD, and B. E. WADDHAM. Respiratory response to inspired CO_2 during acclimatization to an altitude of 12,470. *J. appl. Physiol.* 1957 *11*: 65–71. (37).

KELLOGG, R. H. The role of CO_2 in altitude acclimatization. In: *The regulation of human respiration.* Ed. by D. J. C. CUNNINGHAM and B. B. LLOYD. Oxford, Blackwell Scientific Publ. 1963. 379–395. (33 34).

KELLOG, R. H Central chemical regulation of respiratio I *Handbook of Physiology Respiration vol 1* WASHINGTON, D C. Amer Physiol Soc. 1964 507–534 (10).

KJÄLQUIST Å. and B. K. SIESJÖ The csf/blood potential I sustained acidosis and alkalosi the rat. *Acta physiol. Scand.* 1967 71 255–256. (43 50).

KJÄLQUIST Å., M NARDINI, and B. K. SIESJÖ. Th regulation f extra- and intracellular acid-base parameters in th rat brain during hyper and hypocapnia. *Acta physiol Scand.* 1969 76 485–494 (35 5).

KJÄLQUIST Å. The csf/blood potential in sustained acid-base cha ges in the rat. With calculations of electrochemical potential differences f r H and HCO^- *Acta physiol Scand.* 1970. 78 85–93 (43 45. 46, 49 50 52, fig. 22. 23).

KOCH, L. L. DE. The carotid body system of th higher vertebrates. *Acta Anat (Basel)* 1959 37 265–279 (11).

LAHIRI, S. and J S. MILLEDGE. Acid-base in sherpa ltit de residents and lowlanders at 4880 m. *Resp Physiol* 1967 2 323–334. (40)

LAHIRI, S. Alveolar gas pressures in man with lif -time hypoxia. *Resp. Physiol.* 1968 4 373–386. (37).

LAHIRI, S., F F KAO, T VELASQUEZ, C MARTINEZ, and W PEZZIA. Irreversible bl nted respiratory sensitivity to hypoxia in high altitude n tives. *Resp Physiol* 1969 6 360–374 (27).

LAMBERTSEN, C. J S. J G SEMPLE, M G SMYTH and R GELFAND. H nd Pco_2 as chemical factors in respiratory and cerebral circulatory control. *J ppl Physiol* 1961 16 473–484. (15 21).

LAMBERTSEN, C. J R. GELFAND, and R. A. KEMP Dynamic response characteristics of several CO_2-reactive components of the respiratory control system. I *Cerebrospinal fluid and the regulation f ventilation.* Ed. by C. Mc. C. BROOKS, F F KAO, and B. B. LLOYD. Oxford, Blackwell Scientific Publ 1965 211–249 (21).

LAQUEUR, E. and F V RZAR. Über die spezifische Wirkung der Kohlensäure uf das Atemzentrum *Pflügers Arch ges. Physiol* 1911 143 395–427 (15).

LEE, K. D R. A. MAYOU, and R W TORRANCE. The effect of blood pressure pon chemoreceptor discharge to hypoxia, and the modification f this effect by the sympathico-adrenal system. *Quart J exp Physiol.* 1964 49 171 183 (12).

[illegible]COS, R., H. GAUTIER, d P PASQUIS. Ventilatory oxygen drive in a ute and chronic hypoxia. *Resp Physiol* 1968 4 217 228. (20, 26, [illegible]9)

[illegible]IMANN, G and A. MEESMANN. Über das Bestehen eines Donna gleichgewichtes zwischen Blut und Kammerwasser bzw Liquor Cerebrospinalis. *Pflügers A ch. ges Physiol.* 1924 205 210–232. (35 43).

LENFANT C., K. JOH[illegible]SEN, and D HANSON Bimodal gas exch ge and ventilation-perfusion relationship in lower vertebrates. *Fed. Proc.* 1970. 29 1124 1129 (fig. 1).

LEUSEN, I R. Chemosensitivity of the resprat ry center Influence f CO in the cerebral centricles on respiration. *Amer J Physiol* 1954a. 176 39–44 (9 15).

LEUSEN, I R. Chemosensitivity of the resp ratory center Influe ce f cha ges in the H and total buffer concentrations in the cerebral ventricles o respiration. *Amer J Physiol* 1954b. 176. 45–51 (9 15).

LEUSEN, I R. nd G DEMEESTER. Die hemische Reaktion der Cerebrospi lflüssigkeit im Sauerstoffmangel. *Pflügers Arch ges Physiol* 1960. 270. 390–398 (17)

LLOYD, B. B., M G M JUKES, and D J C C NINGHAM. The relation between lveola oxygen pressure and the respiratory response to carbo d xide in man. *Quart J p Physiol* 1958. 43 214–227 (18, 19).

LOESCHCKE, H H Übe Bestandspotentiale m Gebiete der Med lla Oblongata. *Pflügers Arch ges. Physiol.* 1956a. 262. 517 531 (43)

LOESCHCKE, H H. Über den Einfl ss von CO_2 uf die Bestandspotentiale der Hirnhäute. *Pflügers Arch. ges Physiol.* 1956b. 266 569– 8[illegible] (43)

LOESCHCKE, H. H., and K. H. GERTZ. Einfluss des O_2-Druckes in d r Einatmungsluft auf die Atemtätigkeit des Menschen, geprüft unter Konstanthaltung des al eolären CO_2-Druckes. *Pfl ger Arch. ges. Physiol.* 1958 267 460–477 (17 fig 4)
LOESCHCKE, H. H., B. KATS AOS, and D. LERCHE. Über den Einflu vo Wasserstoffionenkonzentration und CO_2-Druck im Liquor Cerebrospinalis auf die Atmung. *Pfl ger Arch ges Physiol* 1958. 270. 461–466. (15).
MERCKER, H., and M. SCH ... Über Capillarveränderungen des Gehirn bei Höhenanpassung. *Pflügers Arch. ges. Physiol* 1949 251 49 55 (3).
MERCKER, H., and E. OPITZ. Die Gefä se der Pia Mater höhenangepasster K ... inchen. *Pflügers Arch. ges. Physiol.* 1949. 251 117 122. (32).
MILLEDGE, J. S and S. LAHIRI. Respir tory control in lowlanders and Sherpa highlanders at altitude *Resp Physiol.* 1967 2 310–3_2. (19 70 26, 33).
MILLS, E., and McI W Edw rds. Stim lation of aortic and carotid hemoreceptors during carbon monoxide i halation *J appl Phy iol* 1968 25 494 50_ (14).
MINES, A. H., nd S. C. SORE SEN. Ventilatory response to acut hypoxi in goat kids born at high altitude. *Acta ph siol. Scand.* 1969 77 419–441 (79).
MINES, A. H and S. C. SORENSEN. Ventilatory responses of w ke normal goat during acut a d chronic hypoxia. *J ppl Physiol.* 1970 28 826–831 (70 29 37).
MINES, A. H., and S. C SORENSEN. Changes in the lectrochemical potential difference for HCO between blood ad csf and in csf lactat concentration during isocarbic hypoxia. *Acta physiol Scand.* 1971 81 2_3 233 (36 40, 49 51 fig 19 6).
MINES, A. H., C. G MORRILL, a d S. C. SORE SEN. The effect of isocarbic met bolic acidosis in blood on [H] and [HCO⁻] i csf with deductions about the regulation of ctive transport of H /HCO between blood nd csf *Acta physiol Scand* 1971 81 234 45 (49 fig 22 23 24, 25).
MITCHELL, R. A., H. H LOESCHCKE W MASSION, and J W SEVE INGHA US. Respiratory responses mediated through s perficial chemosensitive reas on the med lla. *J appl Phy iol* 1963 18 523–533 (9 15 16).
MITCHELL, R. A. The regulation of respiration in metabolic acidosis nd lkalosis. In *C r bro pl nal fluid and the egul tion of tilation.* Ed. by C. Mc C. B ooks, F F Kao and B B Lloyd. Oxford, Blackw ll Scientific Publ 1965 109–131 (17 20 23 31 36)
MITCHELL, R. A., C. T C RMAN, J W SEVERINGHAUS, B. W RICHARDSON, M M SINGER nd S. SCHNIDER. Stability f cerebrospinal fluid pH in hronic acid-base disturbances in blood *J appl. Physiol* 1965 20 443–452. (35 45 49 fig 1 22, 23)
MOTTSCHALL, H. J and H H LOESCHCKE. Messungen der transmeningealen Pote tials der K ... bei Änderung des CO_2-Drucks und der H Ionen-konzentration im Blut. *Pflüg r A ch ges Physiol* 1963 77 662–670 (35 43)
MOYER, C. A., nd H K BEECHER. Central stimulation f respiration during hypoxia *Amer J Physiol.* 1942. 136 13–21 (36)
NIELSEN, M., and H SMITH. Studies on the regulation of respiration in acu hypoxi Wit an appendix on respiratory co trol during prolonged hypoxia. *A ta physiol scand* 195 293–313 (18 34).
PAPPENHEIMER, J. R., V F NCL, S. R HEISEY and R. D HELD. Role of ce ... of respiration as studied in unanesthetized goats *Amer J Physiol* 19 ... 15 16, 42, 45 53).
P ULI, H G., C V rbürger nd F Reubi. Chronic derangement of cerebrosp nal fluid acid-base components in man. *J appl Physiol.* 1962. 17 993–998. (34)
PLUM, F and J B. POSNER. Blood nd cerebrospinal fluid lactat during hyperventilation. *Amer J Physiol* 1967 212. 864–870. (52).
POLTER, J Untersuchungen an Blutgefässen der Leptomeninx bei kongenitaler Misch agscyanose *Virchow Arch path. Anat* 1956 329 71 91 (32)

Subject Index

The page numbers refer to the present publication and the roman numbers refer to the previous publications listed in f ont of this publication.

Dynamics of Respiratory Adaptation to Muscular

A Systems Analysis

By

OVE WIGERTZ

STOCKHOLM 1971

ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 363

From the Departments of Aviation and Naval Medicine
Faculty of Medicine, Karolinska Institutet,
Stockholm, Sweden

Dynamics of Respiratory and Circulatory Adaptation to Muscular Exercise in Man

A Systems Analysis Approach

By

OVE WIGERTZ

STOCKHOLM 1971

Contents

Contents

This thesis is based on the following papers

I. Wigertz, O Dynamics of ventilation and heart rate in response to sinusoidal work load in man. *J appl Physiol* 1970 *29* 208—218.

II. Broman, S. and O Wigertz, Transient dynamics of ventilation and heart rate with step changes in work load from different load levels. *Acta physiol scand* 1971 *81* 54—74.

III Karlsson, H. and O Wigertz, Ventilation and heart rate responses to ramp-function changes in work load. *Acta physiol scand* 1971 *81* 215—224

IV Bjurstedt, H. and O Wigertz, Dynamics of arterial oxygen tension in response to sinusoidal work load in man. *Acta physiol scand*. 1971 In press.

V Rosenhamer G and O Wigertz, Dynamics of arterial blood pressure responses to sinusoidal work load in man. *Acta physiol scand*. 1971 In press.

In the text these papers will be referred to by Roman numerals I—V

Preface

This work is based on investigations performed during the years 1967—1970 at the Departments of Aviation and Naval Medicine, Faculty of Medicine, Karolinska Institutet.

My profound thanks go to Professor Hilding Bjurstedt, Head of the Department of Aviation Medicine, whose generous support and active interest in the principles of regulation and control in physiology has been of major importance for carrying through this work. I would also like to express my appreciation to Professor Carl Magnus Hesser Head of the Department of Naval Medicine, for constant encouragement, stimulating advice and constructive criticism.

Investigations of the type reported here require close cooperation between research workers in medicine and engineering. I am greatly indebted to my medical colleagues Lennart Fagraeus, Dag Linnarsson, Georg Matell and Gunnar Rosenhamer for amicable cooperation and fruitful discussions. It is also a pleasure to thank my technical colleagues Svetlana Broman, Hans Karlsson, Bertil Lindborg, Mats-Erik Nygårds and Torsten Åström for their assistance in the electronic processing of primary data and for other courtesies too numerous to mention.

I am also especially grateful to Karin Vaster for valuable assistance Iréne Andersson and Barbro Bergström for skilful preparation of data and artistic drawing of diagrams and Alene Eklöf, Gunilla Ramberg, Irène Unander-Scharin and Ylva Zachrisson for capable secreterial assistance.

The work was supported in full by the Swedish Medical Research Council.

Stockholm, January 1971

Ove Wigertz

Abbreviations

V — inspired minute volume (l/min) at ambient temperature and pressure

HR — heart rate (beats/min)

Pa_{O_2} — arterial O_2 tension in mm Hg at 37.0 C

ATP — ambient temperature and pressure

T — indicates the period, i.e. time (in min) for completion of a full sine wave in drive function (work load sinusoid)

T_D — pure time delay (sec)

τ — time constant (sec)

L — value of loss function

Introduction

A major objective of the present work was to analyze the dynamics involved in the adaptation of respiratory and circulatory function to muscular exercise. The nature of the problems tackled chiefly originates from the important thesis that any particular aspect of the behavior of physiological systems ultimately have to be quantitated in order to arrive at an understanding how the homeostasis of the body is maintained in response to varying influences or perturbations. More specifically the work is concerned with the problem how respiratory and circulatory physiological functions involved in the adaptation to muscular exercise are coordinated and controlled to proceed at the right intensity or sequence. In this sense, the subject matter of the present work may be regarded as respiratory and circulatory versions of systems physiology (25) For an engineer facing a physical system that comprises known elements obeying classical physical laws, the system dynamics are usually not difficult to formulate. This may be called the description problem. The physiologist, however must often deal with an existing system whose component properties are incompletely known and sometimes inaccessible for direct measurements during normal operation. The description problem then becomes an inverse or identification problem, *i.e.*, a mathematical model must be derived from empirical input-output measurements (*cf* 12) The above problems, facing the engineer and the physiologist alike, can all be included under the general heading of *systems analysis*

With suitable forcing functions — in this study sine- step- and ramp-function work load patterns — dynamic properties of physiological systems can be studied by analyzing the dynamic characteristics of their responses, *i.e* by determining dynamic relationships between inputs (x) and outputs (y) schematically depicted in Fig. 1

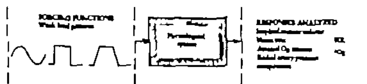


Fig. 1

In this investigation physiological responses to supine, submaximal leg exercise were analyzed using a cycle ergometer with the pedalling rate always kept constant at 60 rpm, irrespective of whether sinusoidal or other work load patterns were applied. The main objectives were:

to obtain quantitative measures (mathematical models) of the dynamic characteristics of the responses of inspiratory minute volume, heart rate, arterial O_2 tension, and arterial pressure in the radial artery by use of 1) time-averaging sinusoidal analysis, 2) step-response analysis and 3) ramp-function testing;

to compare, for ventilation and heart rate, their dynamic behavior as obtained by different work load inputs,

to compare the dynamic behavior of respiratory and circulatory variables in the unsteady state of exercise

to explore the existence of fast (abrupt) components in respiratory and circulatory control during exercise

to examine the interrelations between the responses of arterial pressure and heart rate to exercise.

Methods

Descriptions of the methods and procedures used in the present work have been reported in detail in the separate papers and are therefore only briefly summarized below.

Material

The present results are based on experimental data obtained from 23 healthy young men of whom all were physically active and some were well trained in endurance sports. Some of the subjects participated in more than one of the studies. Dimensional and functional data for each subject have been reported in the respective papers.

Work Load Patterns (Forcing Functions)

All the experimental data were obtained with the subjects in the supine position, resting or performing leg exercise on an electrically braked cycle ergometer. The work load patterns were chosen so as to be well tolerated by the subjects and to avoid any significant accumulation of lactic acid in the arterial blood. Except during the rest periods at the beginning and end of a particular experiment the subject pedalled continuously at 60 rpm. Rest is defined as lying immobile, whereas zero-load pedalling represents pedalling at 60 rpm with the ergometer set at 0 kpm/min. *Sinusoidal work loads* (I, IV, V) were accomplished by replacing the parallel-coupled resistors ordinarily used in the ergometer for constant load exercise by a variable resistor, the slider of which was mechanically linked to a cam-and follower device. The cam was driven by a synchronous motor equipped with a hydraulic variable-speed gear system by which sinusoidal periods in the range 0.75—15.0 min could be accomplished. The contour of the cam was cut for generation of precise sinusoidal work load patterns. The load was varied between the two fixed extremes of 250 and 1050 kpm/min (average work load = 650 kpm/min, amplitude = 400 kpm/min). *Step-function work loads* (II), with the subject pedalling without interruption, were accomplished by remote control and without warning. In this way the changes in work load could not be anticipated by the subject. At the start of the experiment, zero-load pedalling was initiated on verbal command with 3-sec count-down, the flywheel of the ergometer being kept turning to avoid the sudden effort of overcoming flywheel inertia. At the end of each experiment, the subject changed from pedalling to motionless rest following a verbal command. *Ramp function work loads* (III) with pre-set slopes, were obtained as half period triangular waves, using the same modified cycle ergometer with the contour of the cam modified to generate time linear work load patterns. The load was varied between the two fixed extremes of 250 and 1050 kpm/min, the ramp slopes being ± 114, ± 160 and ± 267 kpm · min^{-2}.

Recordings

Inspired minute volume (V) was automatically and continuously computed breath-by breath with a new special purpose digital computer (2) Input signals for the computer were obtained from a Venturi-type low resistance flowmeter (33) and an electronic logic system (19) which provided automatic suppression of extraneous inputs *e.g.* from swallowing. The overall resistance of the flowmeter increases linearly with flow and amounted to 0 45 cm H_2O/l/sec at a flow of 200 l/min. The results of the computations for each breath were clamped and held over the duration of the following breath. *Tidal volume* was obtained by electronic integration of the flow signal, and *respiratory rate* was automatically computed by the aforementioned special purpose computer *Heart rate* (HR) was obtained from chest electrodes and a new linear cardiotachometer (20) operating on the principle of recording the inverted time interval between successive R waves with its value held over the next heart period. *Arterial oxygen tension* (Pa_{O_2}) was recorded continuously in blood drawn anaerobically via an indwelling Teflon catheter in the right or left radial artery The catheter-transducer recording system in cluded a Clark type microelectrode and O_2 monitor (E506, and PHA 927 Radiometer Copenhagen) and was similar to that used by Rosenhamer (26) In the experiments where Pa_{O_2} was recorded, the subjects were given 200 mg Heparin (Vitrum) intrave nously to prevent clotting of blood during its passage from the radial artery through the sampling line and O_2 electrode assembly *Systemic arterial pressure* was recorded from the right or the left radial artery at the wrist, via an indwelling Teflon catheter connected to an electrical manometer (Sanborn, type 267 BC with amplifier, type 311 A) *Radial mean systolic diastolic and pulse pressures* were obtained on-line from the primary pressure signal by use of an analog computer (PACE TR-48) and were recorded sepa rately *Reference sine* and *cosine signals* synchronized with the work load sinusoids were generated with a sine-cosine resolving potentiometer (Helipot Corp., type 5711) powered by a stabilized ±10 V DC supply *Rectal temperature* was recorded by means of a thermistor probe (Yellow Springs Instruments, Inc., No. 401 with amplifier Model 41 TA) *Lactic acid* was determined in samples drawn from a superficial vein in the forearm and analyzed in duplicate by an enzymatic spectro-photometric method (21)

An 8-channel ink recorder (Brush, Mark 200 paper speed 12 mm/min) was used for continuous and simultaneous recording of the variables under study A 14-channel analog (FM) magnetic tape recorder (Ampex FR 100 C, tape speed 1 7/8 ips) was used for storage and subsequent off line analysis of data.

General Experimental Procedure

Before any of the experimental series was started, the subjects had been made familiar with the sensations experienced during supine leg exercise with the specific work load pattern that served as the forcing function. In all experiments, the subject was positioned supine on a bed with his shoulders firmly against fixed support to avoid isometric work with the arms (Fig 2) His shoes were attached with adhesive tape to the pedals with the crank axis at the level of the bed. He then rested for 15—20 minutes before being connected to the recording instruments. The experimental protocols depicting the work load as a function of time appear in Fig. 1 of each separate paper Calibrations and recalibrations of the recording instruments were made as described in the separate papers. Throughout the experiments care was taken to ensure an environment free from disturbing noise or other extraneous influences.

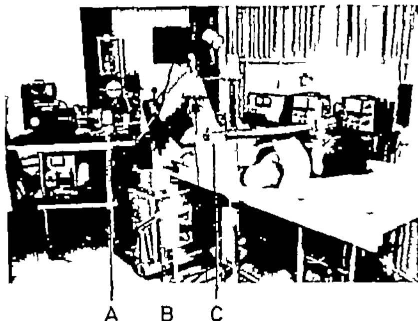


Fig. 2. Subject performing supine leg exercise (I, II III, IV V) A. Function generator for work load profiles. B. Electrically braked cycle ergometer tilted for supine leg exercise. C. Venturi-type flow meter for measurement of inspiratory flow with plastic tubings to differential pressure transducer suspended from cross-bar on vertical stand. For description of the instruments used in the present work, including those not shown in the figure, see Recordings (p. 10)

Off-Line Analysis of Data, Computations

The analysis of experimentally obtained responses of different variables to sine step- and ramp-function work load inputs required the use of dynamic systems identification methods. For this purpose, computer-oriented procedures were designed for estimation of dynamic parameters suitable for coping with physiological variables, which often exhibit considerable spontaneous fluctuations (periodic or not) and random noise.

Thus a procedure for fitting transfer functions of the Laplace type to experimental frequency response data, with the objective to estimate the unknown dynamic parameters of these functions, was developed (I Appendix, for details see [16]). Briefly the procedure included the following stages. For the parameter estimation the criterion selected to obtain the best fit involved minimization of the value of a loss function (L-value). The method includes measures to give the individual data from each period (including the stable-state values) adequate weights in the fitting process. Weighting was also performed to give prominence to the mean dynamic characteristics by suppressing the influence of inter-individual amplitude differences. The computed minimal L values,

which serve as relative indices of the degree of fitting of the selected functions to the experimental data, are smaller the better the fit. They were used to compare the fittings of the first and second-order functions intra individually Furthermore, the L value permitted inter-individual comparisons of the best fits.

Another computer-oriented method was designed for estimation of exponential parameters in experimental step-response data (II Appendix, for details, see [7]) Briefly a two-stage procedure was used, which simultaneously yielded the estimates of the dynamic parameters for the mathematical function adopted. First, the derivative method (24) was applied to the smoothed data to obtain starting values. Subsequently the actual fitting was carried out by applying an iterative least-squares method to the original data, previously subjected only to logical screening (cf Fig. 4) Minimization of the value of a loss function was selected as a criterion of best fit" The normalized loss function (L-value) permitted inter-individual comparisons of best fits. An advantage of this fitting procedure is that it can be carried out without knowledge of the final steady state values. In the present study the first 270 sec of the ventilatory transients, and the first 210 sec of the heart rate transients were processed. These time segments had previously been tested separately for ventilation and heart rate and were found to contain all the essential dynamic information.

Since the application of the procedures summarized above, were made in a statistical framework, involving least squares methods, they also permitted estimation of standard deviations for the computed parameter estimates.

Sine-wave responses

The block diagram I Fig 3 presents the stages involved in the analysis of sine wave response data. Detailed descriptions of the various procedures incorporated are given in the separate papers (I, IV V).

Step responses

In Fig. 4 the general procedure for analysis of step-response data is visualized. The various sub-procedures are described in detail in paper II.

Ramp responses

A schematic representation of the procedure used for analysis of ramp-response data is shown n Fig. 5 Particulars of the analytical procedure are given in paper III.

Correction for signal distortion caused by transducer dynamic response characteristics and by circulatory dispersion

The primary Pa_{O_2} signals were obtained from a catheter-transducer-recording system, the response characteristics of which delayed and damped the intravascular P_{O_2} changes occurring at the catheter tip in the radial artery Mathematical algorithms were derived (for formulas see IV Appendix) for recovery of the Pa_{O_2} changes at the catheter tip by setting-up numerical difference equation, interrelating the input-output signals of the recording system. The parameters (coefficients) of this equation were estimated using repeated step-response curves of the recording system obtained prior to each experiment, and a least squares method. With inserted parameter estimates and after rearrange

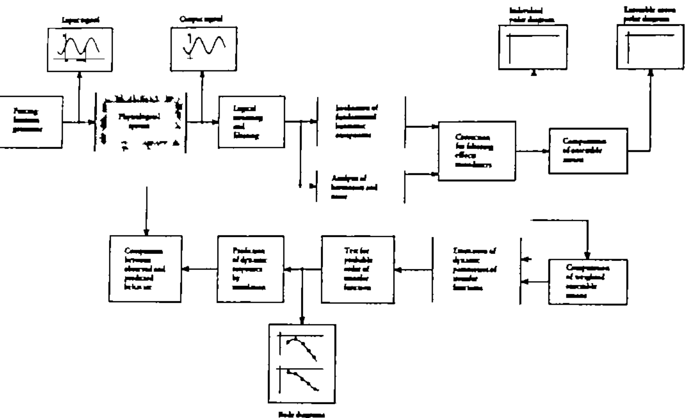


Fig. 3 Flow chart, illustrating the various stages in the analysis of physiological response data with sinusoidal work load forcing. The shaded block represents the physiological system under study

Fig. 4. Flow chart for analysis of step-response data.

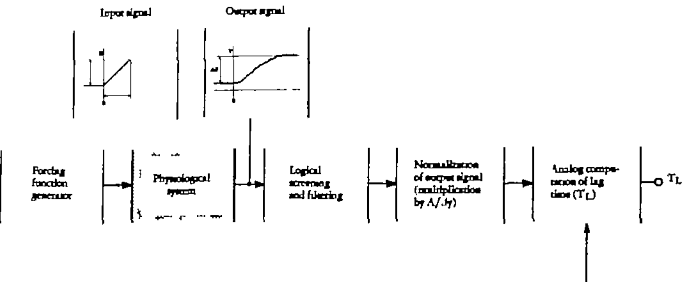


Fig. 5 Flow chart for computation of lag time (T_L) used to characterize the dynamics of ramp-response data. T_L is computed as the time average over the last 1/4 of each ramp according to the formula $T_L = \frac{4}{A} \int_{\frac{3T}{4}}^{T} (x - \frac{A y}{\Delta y})\, dt.$

A is the ramp amplitude (work load difference) attained at time T the slope of the ramp being A/T

ment to describe the input signal as a function of the output signal the numerical equation could be used for reconstruction of P_{aO_2} changes appearing at the tip of the catheter

Delays and damping are also caused by circulatory dispersion as blood traverses the vascular segment between the lungs and the catheter tip. For mathematical recovery of the time courses of P_{aO_2} change in the blood just leaving the lungs and entering the left heart (central arterial P_{O_2}) the same method was applied. The required transfer function for such recovery was obtained from a separate series of experiments (6) For the sinusoidal response data special formulas were utilized, which permitted direct and separate correction of amplitude and phase data.

Results and Discussion

A system is commonly thought to be linear when its response to a given input is always proportional to the magnitude of the input. More specifically a linear system obeys the superposition principle A real system can be linear only within certain limits. However many types of physical and physiological systems or processes (which often include functions having the nature of a negative feed-back) normally operate in a range so as to be nearly linear in their re sponses. Within this range, it should be possible to analyze and understand such systems through the use of linear theory

Dynamic Characteristics of Physiological Responses to Sinusoidal Work Load as Evaluated by Time-A eraging H rmonic Analysis and Mathematical Identification Procedures. — Mathematical Models

The experiments with sinusoidal work load (I IV V) which included input output stable-state measurements and analyses of the contents of harmonics and noise demonstrated the approximate linear behavior of the systems underlying the responses of all variables studied, *viz.* ventilation, heart rate arterial O_2 tension and blood pressure components in the radial artery Linear analysis was therefore applied, and the fundamental components were used in the further evaluation of the basic dynamic behavior of these variables.

Fundamental input-output relationships — Dispersion of primary frequency-response data

From the analyses of the fundamental frequency components in the responses of the different variables to the drive frequencies (periods) used, the individual, primary data for any single variable showed largely uniform response patterns. This was borne out by the polar diagram representations of ensemble-means of amplitudes and phase shifts, in which the small dispersion of individual data could be visualized by plotting the SE.s of the respective rectangular coordinate values (I Fig. 4, IV Fig. 3 V Fig. 4) There were exceptions, however in that the frequency response data for radial mean and diastolic pressures showed relatively large individual variations.

Th Laplace transfer function concept was used to describe the dynamic input output relationship of the fundamental component. It is defined, in the present application, as that function of the complex frequency variable s which, when multiplied by the Laplace transform of the input function of unity amplitude would yield th Laplace transform of the output function. In the present work, th input function was the work load time course and the output functions the corresponding time courses of ventilation, heart rate, arterial O_2 tension and pressure components in the radial artery at the wrist. The transfer functions describe the dynamic characteristics of the responses of these variables in terms of pure time delays, time constants and other parameters. With the harmonic responses of all above mentioned variables presented in the form of polar diagrams it became apparent that the dynamic characteristics of these responses bore a rather close resemblance to those of low-order low pass filters. It therefore was considered justified to select some low-order transfer functions to be fitted to the experimental data, with the objective to estimate the unknown dynamic parameters of these functions using weighting and identification procedures summarized above (see Methods)

For ventilation and heart rate (I) the following transfer functions were tested

$$H(s) = A \quad \exp(-sT_I)/(1 + s\tau) \tag{1}$$

and

$$H(s) = A'' \quad \exp(-sT_D)\,[a_1/(1 + s\tau) + a_2/(1 + s\tau)] \tag{2}$$

where A and A″ are the asymptotic zero-frequency (stable state) amplitudes in the two transfer functions, T_D is pure time delay τ τ_1, and τ_2 the time constants, a_1 and a_2 (a equalling $1 - a_1$) indicate the relative contributions to the amplitude of the two time-constant terms, and s is the Laplace notation of the complex frequency variable. Equation (1) is of the first-order type, whereas equation (2) may be classified as a second-order function (with a zero)

For arterial O_2 tension (IV) a second-order transfer function was chosen which could account for the low-frequency behavior of this variable including a resonance feature

$$H(s) = A'' \quad \exp(-sT_D)\,(1 + s\tau_z)\Big/\left(1 + 2\,\frac{s}{\omega_n} + \left(\frac{s}{\omega_n}\right)^2\right) \tag{3}$$

where τ determines the location of a zero. ζ is the relative damping coefficient, and ω_n is undamped natural angular frequency

The same transfer function was found suitable also for radial systolic and pulse pressures (V) Equation (2) represents a special case of equation (3) *i.e.* when $\zeta > 1$ (overdamped responses)

The graphs to the left in Fig 6—9 are polar representations of (weighted) ensemble-mean amplitudes and phase shifts of *inspired minute volume heart rate central arterial* P_{O_2} *radial systolic* and *radial pulse pressures* (solid circles, corresponding to data represented by triangles and circles in the Bode diagrams appearing in I. Fig. 6 and 7 IV Fig. 5 B V Fig. 5 B and C, respectively) The heavy curves represent the computed best fit transfer functions (for equations, see legends) To the right in Fig. 6—9 are shown the corresponding step responses as predicted from the respective transfer functions using analog computer simulation.

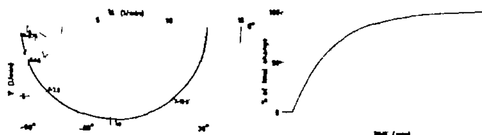


Fig. 6. Dynamics of inspired minute volume in response to sinusoidal work load variations between the extremes of 250 and 1050 kpm/min (11 subjects)

Left: Amplitudes and phase shifts in polar representation. *Solid circles* observed ensemble-mean values (weighted, *cf* data in I. Fig. 6) *Heavy curve* best fit transfer function as given by

$$H(s) = 12.7 \exp(-2.6\,s)/(1 + 69.9\,s) \quad \text{l/min ATP}$$

Right: Corresponding normalized time response to step change i work load predicted from frequency-analysis data using the above transfer function (T_D omitted)

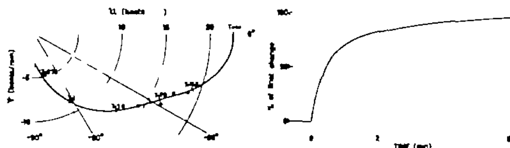


Fig. 7 Dynamics of heart rate in response to sinusoidal work load variations between the extremes of 250 and 1050 kpm/min (11 subjects)

Left: Amplitudes and phase shifts in polar representation. *Solid circles* observed ensemble-mean values (weighted, *cf* data in I Fig. 7). *Heavy curve* best fit transfer function as given by

$$H(s) = 22.6 \exp(-0.9\,s)\,[0.63/(1+21.1\,s) + 0.37/(1+147.3\,s)]$$

beats/min

Right: Corresponding normalized time response to a step change in work load predicted from frequency-analysis data using the above transfer function (T_D omitted)



Fig. 8. Dynamics of Pa_{O_2} in blood leaving the lungs and entering the left heart (central arterial P_{O_2}) in response to sinusoidal work load variations between the extremes of 250 and 1050 kpm/min (7 subjects).

Left: Amplitudes and phase shifts in polar representation. *Solid circles* observed ensemble-mean values (*cf* data in IV Fig. 5 B) *Heavy curve*, best fit transfer function as given by

$$H(s) = -3.4 \exp(-15.3\,s)\,(1+132\,s)/\left(1 + 2.2\,\frac{193\,s}{2\pi} + \left(\frac{193\,s}{2\pi}\right)^2\right)$$

mm Hg

Right: Corresponding normalized time response to a step change in work load predicted from frequency-analysis data using the above transfer function. Note time delay (15.3 sec) between onset of step change in work load at zero time and the beginning of drop in central arterial P_{O_2}.

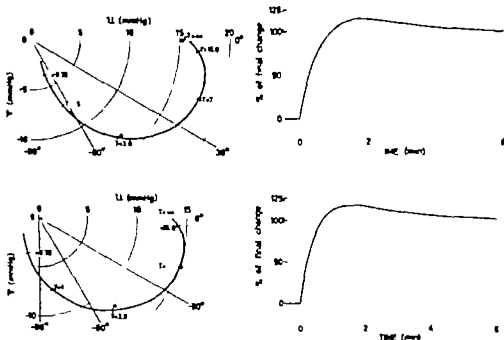


Fig. 9 Dynamics of pressure components in *adial artery* in response to sinusoidal work load variations between the extremes of 250 and 1050 kpm/min (7 subjects)

Left: Amplitudes and phase shifts f *adial systoli* (above) and *adial pulse pressure* (below) in polar representation. *Solid circles* observed ensemble-mean values (weighted cf data in V Fig. 5). *Heavy curves* best fit transfer functions as given by:

$$H(s) = 15.3 \exp(1.4\,s)\,(1 + 103\,s) \Big/ \left(1 + 1.9\,\frac{5.7\ 60\,s}{2\pi} + \left(\frac{5.7\ 60\,s}{2\pi}\right)^2\right)$$

mm Hg (systolic pressure)

$$H(s) = 12.5 \exp(-4.0\,s)\,(1 + 134\,s) \Big/ \left(1 + 2.2\,\frac{5.7\ 60\,s}{2\pi} + \left(\frac{5.7\ 60\,s}{2\pi}\right)^2\right)$$

mm Hg (pulse pressure)

Right. Corresponding normalized time responses to a step change in work load predicted from frequency-analysis data using the above transfer functions (T_D omitted)

Dynamics of Ventilation and Heart Rate in Response to Step-Function (Positive and Negative) and Ramp-Function Forcings

When the ventilatory and heart rate step responses (II) were visualized using the Brush recorder it became apparent that they resembled low-order exponential time functions. To define more accurately their time courses, the transient responses were subjected to dynamic analysis by adopting the following first and second-order functions, $f(t)$ and $f_r(t)$ with the objective of estimating their unknown dynamic parameters using the procedures related above (see Methods)

$$f(t) = a_0 + a_1 \exp[-(t - T_D)/\tau] \quad (4)$$

$$f_r(t) = a_0 + a_1 \exp[-(t - T_D)/\tau_1] + a_2 \exp[-(t - T_D)/\tau_2] \quad (5)$$

a_0, a_1 and a_2 are amplitude coefficients, τ_1 and τ_2 time constants, and T_D is pure time delay. $f(t)$ and $f_r(t)$ are the solution curves to low-order linear differential equations for which the roots of the characteristic equations are real, distinct and negative when subjected to a step-input forcing function. It may be noted that equations (4) and (5) are step responses of systems having the Laplace transfer functions (1) and (2) with equivalence prevailing for the coefficients a_1 and a_2.

In order to estimate the dynamic parameters (time constants) in the ramp responses of ventilation and heart rate an attempt was initially made to use a difference-equation identification procedure. It was found that first-order functions were obtainable for both variables whereas the estimation procedure for second-order functions failed to converge. Thus, no conclusions could be drawn as to the order or slope-dependence (rate-sensitivity) of the responses using this approach. It is well known that ramp inputs are not well suited for dynamic parameter identification especially when the response data are contaminated by noise, see *e.g.* Strobel (30). The method used (III Appendix) however permitted the computation of a compound measure of system dynamics in the form of lag time (T_L) and also the exploration of the existence of slope-dependence.

The main results from the analysis of the ventilation and heart rate responses to step-function and ramp-function forcings are described in the following section in terms of time constants, pure time delays and lag times (T_L) respectively.

Comparisons between the Dynamics of Ventilation and Heart Rate in Response to Different Work Load Inputs

One of the objectives of the present work was to obtain quantitative measures of the dynamic properties of the systems underlying the responses of ventilation and heart rate to different types of work load inputs. In the following a brief

account is given of the extent to which the parameter estimates obtained for these variables were influenced by the application of sine- step- and ramp-function forcings.

Time constants

From the experiments with sine-function forcing (I) mathematical parameter estimation yielded a first-order transfer function for the ventilatory response with a time constant of 70 ± 6 sec (I Table 4 A) No statistically significant improvements of individual L-values could be obtained with second-order fitting.

For the heart rate data it was found that a better fit was obtained with the second-order than with the first-order transfer function. For the mean frequency response data the L-value was lowered by as much as 13 5 times in favor of the second-order fitting· by applying statistical F-test it could be shown that this reduction was significant. The estimated time constants were 21 ± 2 sec and 147 ± 21 sec, respectively with the faster component accounting for 63 % of the total response. The superior fit of the second-order function can be illustrated by plotting both the first and second-order best fit transfer function in the Bode diagram representing the experimental data (I Fig. 7)

The experiments with step-function forcings (II) included positive and negative step changes of the same magnitude (650 kpm/min) initiated from different work levels, viz. positive steps from 0 (loadless pedalling) 300, and 650 kpm/min, and negative steps from 650 950 and 1300 kpm/min. By fitting low order exponential functions corresponding to the transfer functions used for the frequency-response data (p. 18) it was found that first-order functions yielded a satisfactory degree of fitting to the experimental mean transient responses of the ventilation. The first-order best fit function for the ventilatory response to the positive work load step 300 → 950 kpm/min yielded a time constant of 67 ± 2 sec (II. Table 4, Fig. 3) It may be noted that this value is very close to that obtained with sinusoidal work load for about the same work load region. When the second-order exponential function was fitted to the mean transient responses for ventilation, application of Cholesky's orthogonality test showed that the order of the function was too high.

The mean transient responses of the heart rate regularly showed a fast initial phase followed by a slow adjustment to the final level, suggesting that an exponential function with at least two time constants might be required for accurate description. When the initial work level was increased from 0 kpm/min to 300 and 650 kpm/min, the estimated time constants for the fast com-

ponent of the positive step responses remained essentially unchanged (range 9 0—11 7 sec) while those of the slow component varied between 1.8 and 3 7 min The share of the slow component in the total response increased with the initial work level and amounted to about 19 30 and 47 % respectively Corresponding results were obtained with negative step changes. The time constants obtained for the heart rate response to the positive work load step 300 → 950 kpm/min, 12 ± 1 and 133 ± 35 sec were closely similar to those obtained with sinusoidal work load, the faster component accounting for 71 % of the total response.

In the experiments with ramp-function forcing (III) the analysis of the ventilatory and heart rate responses was made by determining their T_L values (lag times see p 22) For ventilation it was shown that the lag time, which for a first-order system with no time delay corresponds to the time constant (III Appendix) averaged 66 sec for all ramp responses. This values agrees well with the time constants given above for the ventilatory responses to sine- and step-function forcings. There was no indication that the ventilatory responses were influenced by varying the slope of the ramp, whether positive or negative

For heart rate, on the other hand, both the individual and group-mean T_L values were dependent on the slope of the ramp Thus, for the positive and negative ramp slopes of 114, 160 and 267 kpm min^{-2} the group-mean T_L values were 84 56, and 39 sec, respectively This demonstrated a significant shortening of the T_L values when the ramp became steeper Granted second order dynamics of the heart rate responses to work load, as found with sine and step-function work loads, this suggests that the share of the shorter time constant becomes larger the steeper the ramp

Pure time delays

In the experiments with sine-function forcings (I) the weighted ensemble mean T_D-value for ventilation (first-order transfer function) was 2 6 sec, and for the heart rate (second-order transfer function) 0 9 sec (I Table 4 A and B) After subtraction of the pure time delays inherent in the recordings of ventilation and heart rate, here assumed to be approximate 3.0 and 0 5 sec, respectively true T_D s should average — 0 4 and 0 4 sec.

With step-function forcings the true T_D s for weighted ensemble-mean responses ranged between — 2.6 and 7 1 sec for ventilation, and between — 1.5 and 3 5 sec for heart rate (for primary values, see II Table 4 and 5 and 8 respectively) these values refer to all positive and negative steps when classed together For the positive work load step 300 → 950 kpm/min, the corresponding T_D-values were 4.0 sec for ventilation and 1 5 sec for heart rate In this

connection, it should be noted that the step-response T_D's showed much larger variations than the T_I's obtained by harmonic analysis, presumably due to overlying, largely random, fluctuations in the variables under study: these fluctuations influenced the extraction of T_I's especially in the automatic fitting procedure used for step-response analysis. Even so, the time delays obtained can be regarded as relatively unimportant for the description of the adjustments of ventilation and heart rate to step changes in work load. In this sense, there was a clear agreement with the results from the experiments with sine-function forcings.

Some Aspects of Respiratory and Circulatory Control during Exercise as Derived from the Present Work

It is now generally held that the changes in cardiac output in the steady state of supine leg exercise occur primarily through adjustments in the heart rate, the stroke volume remaining relatively constant except at heavy or exhaustive work (4 5 14 26, 27 31 32) Jones *et al* (15) have recently shown that this is the case also during the transient phases of supine exercise. In the present work (I—V) with the subjects exercising in the supine position, the time courses of the changes in cardiac output should therefore be similar to those in heart rate. In the following sections, the dynamics observed or predicted for heart rate will therefore be assumed to be similar to those for cardiac output.

Dissociated behavior of respiratory and circulatory responses

The main differences that were observed with regard to the dynamics of ventilation and heart rate (cardiac output) with sine and step-function work load inputs can be summarized as follows 1) Whereas the responses of ventilation could be accurately described by first-order models, those of heart rate required second-order models. 2) The ventilatory responses always lagged behind those of the heart rate (cardiac output) with sinusoidal work load as well as with positive and negative step changes in work load irrespective of the initial work level.

The ventilatory and heart rate ramp responses likewise exhibited clear differences in that the parameter T_L (lag time) used to define the time by which the ramp response in its stationary condition lags behind the work load input signal showed that the heart rate response was clearly dependent on the slope of the ramp, whereas the ventilatory response was largely independent

of the slope. In addition, it was observed that with the steepest ramp, 267 kpm min^{-2} the ventilatory response lagged considerably behind that of the heart rate (cardiac output)

In earlier investigations, Stegemann (28, 29) employed sinusoidally varying work load to study the frequency responses of ventilation and heart rate. For ventilation, this author found a phase lag approaching 180 degrees at a period of 30 sec, which was ascribed to the combined effect of the phase lag of a first order system and a pure time delay corresponding to the leg muscle-to-brain circulation time. However this interpretation seems questionable, since it relied on data in a high frequency region where the responses must conceivably have been obscured by noise and harmonics. For heart rate, Stegemann compared the frequency response with that of a first-order electrical RC-analog with a time constant of 27 sec. No attempt was made to fit second-order models, and consequently no conclusion could be made as to the order of the transfer functions for either variable.

The observation that the ventilation lagged behind the cardiac output in the unsteady-state exercise tested (*cf* *e.g.* I Fig. 8) clearly indicates that in exercise the respiratory and circulatory control systems exhibit dissociated dynamic properties.

The above mentioned findings raised the question of the dynamic behavior of arterial O_2 tension in unsteady-state exercise Time-averaging harmonic analysis of this variable, as recorded in the radial artery at the wrist, showed that the dynamic P_{aO_2}/work load relationship could be described by a second-order transfer function with a marked resonance peak for periods near 3.0 min and an estimated pure time delay of 29 sec. By correcting for the time delay and damping involved in the passage of blood from the pulmonary end-capillaries to the catheter tip in the radial artery (6) the delay after which a change in work load was reflected by a change in central arterial P_{O_2} could be estimated to 15 sec, a positive step in load then resulting in a transient drop in P_{aO_2} (*cf* Fig. 8, right) The dynamic behavior of central arterial P_{O_2} (Fig. 8) must conceivably be an expression of the dissociated dynamic properties of the respiratory and circulatory control systems. The estimated 15 sec pure time delay for the central arterial P_{O_2} response suggests that the onset of changes in one or more variables critical for the dynamics of the pulmonary O_2 exchange occurs in the lungs some 15 sec after a change in work load. Since the changes in both the ventilation and heart rate (cardiac output) exhibit negligible time delays and, furthermore, the alveolar-arterial O_2 difference can be assumed to remain approximately constant, the most likely explanation for the estimated time delay seems to be that the $a-\bar{v}$ O_2 difference did not change appreciably for a corresponding period of time. One explanation that presents itself immediately

is that the 15 sec delay was due to the leg muscle-to-lung circulation time An alternative interpretation is that part of this delay occurred as a result of a de layed onset of change in the local a—v O_2 difference in the working muscles.

The harmonic analysis of the ventilatory and heart rate responses demon strated that both variables showed low pass filtering properties often seen with variables assigned to controlling functions in physical closed loop control systems. By contrast, th. analysis of the P_{aO_2} frequency response exhibited the characteristics of a regulated variable (regulated implying a variable held within narrow limits in the steady state condition)

Abrupt components in respiratory and circulatory control mechanisms

The step responses of ventilation and heart rate (cardiac output) exhibited negligible time delays, indicating that early readjustments of these variables were under neurogenic influence. Their frequency responses, on the other hand, show ed a progressive decrease in amplitude with increasing input frequency suggesting that their control was unaffected by any existing fast (abrupt) component of cerebral or mechanoreceptive origin.

The existence of an abrupt component in exercise hyperpnea has long been a matter of debate (1 3 8, 9 10 11 18, 23) In the present study the early ventilatory responses to step changes in work load were gradual. However abrupt changes in ventilation regularly occurred with the transitions from rest to loadless pedalling at the beginning (II Fig. 6) and from zero-load pedalling to rest at the end of the experimental protocol. Thus, the demonstration of an abrupt component in dynamic exercise hyperpnea (*cf* 10) may require that motionless resting serve as the control condition. Similarly the most abrupt changes in heart rate were seen when loadless pedalling started or ended with motionless resting as the control condition (II Fig. 5 and 6) Since loadless pedalling was always started and stopped on verbal command, it cannot be settled whether these abrupt responses of ventilation and heart rate were manifestations of conditioned reflexes associated with the countdown, or of other neurogenic influence of cerebral and/or peripheral mechanoreceptive origin. With the positive steps in work load initiated from different load levels and with the subjects already pedalling, the rise in heart rate was usually preceded by an initial short lasting (2—4 sec) drop which tended to become less marked the higher the initial work load (II Fig. 5) The cause of this initial drop in heart rate, which did not occur with transition from rest to loadless pedalling, requires further study

Interrelations between the responses of arterial pressure and heart rate

Using the results obtained by time-averaging harmonic analysis of the responses of the heart rate, on one hand, and the radial systolic and radial pulse pressure on the other (graphically represented in Fig. 7 and 9) an attempt was made to 1) estimate central pressure responses, and 2) explain the overdamped response of the heart rate (V) The transfer functions for central systolic and pulse pressures were estimated using gain constants obtained from comparisons between central and peripheral arterial pressure components (17) These gain constants were obtained from measurements with the opening of the catheters directed against the blood flow Consequently the velocity pressure was included in the pressure measurements. Whereas this pressure component is negligible (less than 1 mm Hg) in the radial artery with inactive arm muscles, it can become considerable in the aorta even during moderate leg exercise Further, if the fluctuations in heart rate are assumed to parallel those in cardiac output and hence those in the mean velocity of the blood in the proximal aorta, the share contributed by the velocity pressure to the total pressure increases in proportion to the heart rate squared. This in turn means that, as the heart rate (cardiac output) increases, there would be a *progressively increasing difference* between the total pulse pressure centrally and the component thereof which distends the walls of the central vessels (lateral pressure) Consequently the lateral pulse pressure would show a more marked overshoot than does the total pulse pressure. In contrast to the systolic and pulse pressures, the time response of the heart rate is overdamped (Fig. 7) Reflex slowing of the heart is known to result from stimulation of the arterial baroreceptors, this effect probably originating from increased pulse pressure rather than increased mean pressure (*cf* 13) If so, the observation of an overshoot in the pulse pressure might, at least in part, explain the occurrence of an overdamped heart rate response. If the velocity component of the pulse pressure is not detected by the baroreceptors (*cf* 22) a notion that can be questioned, then the overshoot of the lateral pulse pressure would have a stronger damping effect on the heart rate increase than would be expected from the time response of the total pulse pressure.

The changes in radial diastolic pressure were small and not clearly related to the drive frequency The response of the radial mean pressure could not be described accurately by either first or second-order functions, but exhibited phase lead features, suggesting sensitivity to rate of change in work load.

General Summary

1 The aim of the present investigation was to examine the dynamic characteristics of the responses of the following variables to submaximal supine leg exercise in man, viz. inspired minute volume, heart rate, arterial O_2 tension, and systolic, diastolic, mean, and pulse pressures in the radial artery at the wrist. Healthy physically active men served as test subjects. A systems analysis approach was used in the sense that advantage was taken of recently developed, and new methods for analysis of technical processes and control systems, and that response characteristics of physiological systems were identified and quantified in mathematical terms. Work load forcings of the sine- step- and ramp-function type were used variations in work load were between the extremes of 0 (zero-load pedalling) and 1300 kpm/min.

2. Time-averaging harmonic analysis showed a clear predominance of the fundamental component over the second and third harmonics in the responses of all the above-mentioned variables, indicating approximately linear properties of the underlying systems within the work load regions studied.

3 With harmonic analysis the weighted ensemble-mean ventilatory response to sinusoidal work loads yielded a transfer function which was of the first order with an estimated time constant of 70 ± 6 sec (p. 23) The corresponding transfer function for heart rate was of the second-order type with time constants of 21 ± 2 and 147 ± 21 sec, the faster component accounting for 63 % of the total response (p. 23) Both for the responses in ventilation and in heart rate to sinusoidal work load, pure time delays were negligible.

4. The sinusoidal respone of arterial O_2 tension could be described by a second order transfer function (p. 20) with a marked resonance peak in amplitude for periods near 3.0 min (peak-to-peak deviations approaching 14 mm Hg) This transfer function exhibited characteristics of a variable regulated so as to be held within narrow limits. It also indicated that the basic response of P_{aO_2} to a change in work load is a change in the opposite direction and that with step changes in work load the response in P_{aO_2} appears as a temporary over shoot. When referred to blood entering the left heart, the response of P_{aO_2} occurred after a time delay of approximately 15 sec.

5 The sinusoidal response of the systolic and pulse pressures in the radial artery could be described by second-order transfer functions (p. 21) Resonance

occurred for periods near 7.0 min, corresponding to overshoots for both variables in the time domain. Using known characteristics of the peripheral distortion of the two pressure components, these functions, with the exception of an amplification factor could be regarded as approximately representative also for the corresponding pressures centrally The changes in radial diastolic pressure were small and not clearly related to the drive frequency The response of the radial mean pressure could not be described accurately by either first or second-order functions, but exhibited phase lead features, suggesting sensitivity to rate of change in work load.

6 With a step-function change from 300 to 950 kpm/min, ventilation was found to follow a mono-exponential pattern with a time constant which was close to that obtained with sinusoidal work load, (67 ± 2 sec, p. 23) The heart rate response required a second-order exponential function, with time constants again similar to those seen with sinusoidal work (12 ± 1 and 133 ± 35 sec, p 24) the faster component accounting for 71 % of the total response. With ramp function work loads, the ventilatory response showed largely similar characteristics as those obtained with sine- and step-function work load forcings (p 24) Heart-rate ramp responses were found to be more rapid the steeper the ramp (p. 24) Granted second-order dynamics of the heart rate response to work load, the slope dependence of the response suggests that the share of the shorter time constant becomes larger the steeper the ramp (p 24) As with sinusoidal forcings, the ventilation and heart rate responses to step changes in work load were associated with negligible time delays, indicating that the early readjustments in both variables were under neurogenic influence.

7 Since the subjects were always exercising in the supine position, the dynamic characteristics observed for the heart rate responses can be assumed to reflect those in cardiac output. The results therefore indicate that in exercise the respiratory and circulatory control systems exhibit dissociated dynamic properties, readjustments in ventilation always lagging behind those in cardiac output (p 25) It was further concluded that the ventilation and heart rate responses to sinusoidal or step changes in work load (with the subjects already pedalling) were unaffected by any existing, fast (abrupt) component of cerebral or peripheral mechano-receptive origin, or both. Evidence was obtained, that the demonstration of an abrupt component in dynamic exercise hyperpnea may require that motionless resting serve as the control condition (p 27)

Refer... –

1. ASMUSSEN, E. and M. NIELS... the transition from rest to work ... 16. 270—285

2. ASTRÖM, T. and O. WIGERTZ, ... breath calculation of respiratory ... Inst. Stockholm, March 1966. 23 ...

3. BEAVER, W. L. and K. WASSERMAN ... end of exercise. J. appl. Physiol. ...

4. BEVEGARD, S., A. HOLMGREN ... on the circulation at rest and during ... on the stroke volume. Acta physiol. scand. ...

5. BEVEGARD, S., A. HOLMGREN ... trained athletes at rest and during ... volume and the influence of body ...

6. BJURSTEDT, H., H. KARLSSON ... monary capillary-to-radial artery ... rest and exercise. Report Lab. ... 1970. 15 pp.

7. BROMAN, SVETLANA and O. WIGERTZ, ... meter estimation in physiological ... Aviat. Naval Med. Karol. Inst. Stockholm ...

8. CRAIG, F. N., E. G. CUMMINGS and ... at the beginning of exercise. J. appl. Physiol. ...

9. DEJOURS, P. La regulation de la ven... chez l'homme. J. Physiol. (Paris) 1959 51 ...

10. DEJOURS, P. Control of respiration in ... logy. Respiration. edited by W. O. Fenn and ... Handbook ... Physiol. Soc. 1964. 3. I. 631—648.

11. DEJOURS, P. Neurogenic factors in the ... Circulat. Res. 1967. 20. Suppl. I. 146—153

12. GRODINS, F. S., General applicability of ... some exemplification in the respiratory system ...

13. HEYMANS, C. and E. NEIL, Reflexogenic ... Churchill, London. 1958. 271 pp.

14. HOLMGREN, A., Circulatory changes during ... Lab. Invest. 1956. 8. Suppl. 24. 1—97

15. JONES, W. B., R. N. FINCHUM, R. O. RUSSELL ... T. J. REEVES, ... cardiac output response to multiple levels of ... J. appl. Physiol. 28. 183—189

16. KARLSSON H. and O WIGERTZ, Identification of the dynamics of physiological systems. fitting linear transfer functions to experimental harmonic analysis data. *Report Lab Aviat Naval Med Karol. Inst Stockholm.* Aug. 1970 16 pp.

17 KROEKER, E. J and E. H WOOD Comparison of simultaneously recorded central and peripheral arterial pressure pulses during rest, exercise and tilted position in man. *Circulat Res* 1955 *3* 623—632.

18 KROGH, A. and J LINDHARD The regulation of respiration and circulation during the initial stages of muscular work. *J Physiol (Lond)* 1913 *51* 112—136.

19 LINDBORG B. and O WIGERTZ, An electronic logic system for recording respiratory functions. *Report Lab Aviat Naval Med Karol. Inst Stockholm.* March 1966. 10 pp.

20 LINDBORG B., O WIGERTZ and Th. ÖDMAN A beat-to-beat heart rate meter with linear analog output for muscular exercise studies. *Report Lab Aviat Naval Med Karol. Inst Stockholm.* Dec. 1969 23 pp.

21 LUNDHOLM, L., E. MOHME-LUNDHOLM and N VAMOS, Lactic acid assay with L (+) lactic acid dehydrogenase from rabbit muscle. *Acta physiol. scand* 1963 *58* 243—249

22. MARX, H. J L. B. ROWELL, R. D CONN R. A. BRUCE and F KUSUMI, Maintenance of aortic pressure and total peripheral resistance during exercise in heat. *J ppl Physiol* 1967 *22* 519—525

23 MATELL, G Time-courses of changes in ventilation and arterial gas tensions in man induced by moderate exercise. *Acta physiol. scand* 1963 *58* Suppl. 206. 1—53

24. PERL, W A method for curve fitting by exponential functions. *I t. J appl. Radiat and Isotop* 1960 *8* 211—222

25 Physiology Training Committee, National Institute of General Medical Sciences. A view of systems physiology *Physiologist* 1968. *11* 115—133

26. ROSENHAMER, G Influence of increased gravitational stress on the adaption of cardiovascular and pulmonary function to exercise. *Acta physiol scand* 1967 *68* Suppl. 276. 1—61

27 RUSHMER, R. F Postural effects on the baselines of ventricular performance. *Circulation.* 1959 *20* 897

28. STEGEMANN J Beitrag zur Dynamik der Atmungsregelung bei leichter Muskel arbeit. *Z. Biol* 1958. *110* 449—456.

29 STEGEMANN J Zum Mechanismus der Pulsfrequenzeinstellung durch den Stoffwechsel. IV Zur Frage der Lokalisation der stoffwechselempfindlichen Muskelreceptoren *Arch Ges Physiol* 1963 *276*. 511—524.

30. STROBEL, H., On the limits imposed by random noise and measurement errors upon system identification in the time domain. *Proceed ngs IFAC Symposium — Ident f cation in Automatic Control Systems* — Prague. 1967

31 WADE, O L. and J M. BISHOP *Cardiac Output and Regional Blood Flow* Blackwell Scientific Publications, Oxford. 1962. 268 pp.

32. WANG Y R. J MARSHALL and J T SHEPHERD The effect of changes in posture and of graded exercise on stroke volume in man. *J clin Invest* 1960. *39* 1051—1061

33 WIGERTZ, O., A low-resistance flow meter for wide-range ventilatory measurement. *Respir Physiol* 1969 *7* 263—270

SUPPLEMENTUM 364

ACTA PHYSIOLOGICA SCANDINAVICA

C. Hildebrand and S Skoglund

Calibre spectra of some fibre tracts in the feline central nervous system during postnatal development.

C. Hildebrand

Ultrastructural and light microscopic studies of the nodal region in large myelinated fibres of the adult feline spinal cord white matter

Ultrastructural and light microscopic studies of the devel oping feline spinal cord white matter I. The nodes of Ranvier

Ultrastructural and light microscopic studies of the developing feline spinal cord white matter II Cell death and myelin sheath disintegration in the early postnatal period

C. Hildebrand and S Skoglund

Histochemical studies of adult and developing feline spinal cord white matter

STOCKHOLM 1971

From the Department of Anatomy Karolinska Institutet
S—104 01 Stockholm 60 Sweden

SUPPLEMENTUM 364

ACTA PHYSIOLOGICA SCANDINAVICA

C. Hildebrand and S Skoglund

Calibre spectra of some fibre tracts in the feline central nervous system during postnatal development

C Hildebrand

Ultrastructural and light microscopic studies of the nodal region in large myelinated fibres of the adult feline spinal cord white matter

Ultrastructural and light microscopic studies of the developing feline spinal cord white matter I The nodes of Ranvier

Ultrastructural and light microscopic studies of the developing feline spinal cord white matter II Cell death and myelin sheath disintegration in the early postnatal period.

C. Hildebrand and S Skoglund

Histochemical studies of adult and developing feline spinal cord white matter

STOCKHOLM 1971

Kungl. Boktryckeriet P. A. Norstedt & Söner
Stockholm 1971
713070

Contents

Calibre Spectra of Some Fibre Tracts in the Feline Central Nervous System During Postnatal Development

By

Claes Hildebrand and Sten Skoglund

Introduction

It has been proposed that the functional capacity of developing central and peripheral fibres should be directly influenced by the appearance of myelin (see Tilney and Casamajor 1924). On this basis several reports describe the sequence of myelination in the central and peripheral nervous system (Langworthy 1929, Windle *et al.* 1934, Fox *et al.* 1967, to mention a few). However the works of Purpura *et al.* (1964) and Conway *et al.* (1969) on the developing corticospinal tract clearly demonstrated a lack of direct relationship between early myelination and any major change in conduction velocity of central fibres. This view is also supported by the results of Windle *et al.* (1934) who did not find any significant functional improvement correlated with the initial deposition of myelin. That the same situation is at hand also in developing peripheral nerves seems very likely (see Gutner 1936, Rushton 1951) in spite of the reports of Carpenter and Bergland (1957) and Bergland (1960) who in the chicken sciatic nerve found that the most pronounced increase in conduction velocity occurred during the initial myelination.

A number of authors have applied fibre size determinations to the developing peripheral nervous system (Duncan 1934, Rexed 1944, Mohiuddin 1951 among others). In 1965 Skoglund and Romero published a systematic investigation of the morphological maturation of feline spinal nerves and roots measuring the fibre sizes at different ages. From this and earlier works it was evident that a close correlation between fibre size and functional properties is at hand in developing peripheral nerves (see Skoglund 1966). Since then a number of studies have been published which, along the same lines, correlate physiological properties to fibre size in a wide range of developing peripheral nerves (Nyström and Skoglund 1965, Ekholm 1967, Nyström 1968, Schwieler 1968).

The postnatally occurring ultrastructural and histochemical changes in the nodal-paranodal region of spinal root fibres fit very well into this develop-

mental scheme (Berthold and Skoglund 1965 1967 1968 a, b Berthold 1968 a) Thus with increasing fibre size the nodal paranodal ultrastructural and histochemical appearance changes towards increasing complexity up to a fibre size around 4 μ when the fibre attains close to adult functional properties (Skoglund 1966) In order to find out whether a correlation between structure and function similar to that found in peripheral nerves is also present in the central nervous system calibre spectra of some central tracts were studied during postnatal development.

Material and methods

The brainstem and cervical spinal cord of 7 adult cats and 25 kittens of varying ages (1 day—6 months) were fixed by vascular perfusion. The animals were first anaesthetized with 20—40 mg/kg sodium pentobarbital, tracheotomized and artificially respired with 100 % oxygen The surgical preparation of the animals before perfusion was similar to that described by Palay *et al.* (1962) A teflon cannula was inserted through an incision in the left ventricle into the ascending aorta, the right atrium was cut open and perfusion started. Initially a warm (37 C) Ringer solution containing 2.7 % Rheomacrodex (Pharmacia, Sweden) was administered at a pressure around 100 mm Hg Simultaneously the descending aorta was clamped at the level of the diaphragm. The Ringer solution was followed by the fixative which was instilled at the same temperatures, pressures and quantities described by Berthold (1968 b) The fixative solution consisted of 5 % glutaraldehyde in a 300 mOsm Millonig buffer with addition of 2.7 % Rheomacrodex (Berthold 1968 b)

In younger animals the whole spinal cord usually became adequately fixed Useful specimens were then obtained both of cervical and lumbar spinal cord white matter in one and the same animal, which is a prerequisite for the comparison of the lumbar and cervical stage of development at a certain age. In the older animals this method frequently failed to preserve the lumbar spinal cord white matter although it was possible to obtain usable lumbar specimens in some cases. Therefore the lumbar spinal cord was perfused in some animals as described by Berthold (1968 b)

After laminectomy the brainstem and the first cervical segment of the spinal cord were removed. The first cervical segment was cut into transverse slices and each slice was divided into three parts as seen from Fig 1 A. The pyramid was dissected out and transversely sliced so that specimens from above the decussation with an appearance as in Fig 1 B were obtained In the lumbar spinal cord the dorsal funiculus of the seventh lumbar segment was cut free

and transversely sliced After postfixation in the fixative lacking Rheomacrodex, for 4 hours the specimens were rinsed in a 300 mOsm veronal-acetate or Millonig buffer postosmicated in a solution of 2 % osmium tetroxide in the veronal acetate buffer rinsed again in the veronal-acetate buffer and dehydrated in acetone before embedding in Vestopal W (Ryter and Kellenberger 1958) Semithin (0.5—1 0 μ) transverse sections were cut with a LKB 4801 *Ultrotome and stained with toluidine blue* (*Maunsbach et al* 1962) For the light microscopic examination and photographing a Leitz Wetzlar photomicroscope supplied with Kodak Panatomic X rollfilm was employed. The areas of white matter to be used for calibre spectrum measurements were selected as described below

1 The dorsal columns

The most dorsomedial part in each of the gracile and cuneate fasciculi in the first cervical segment was identified and photographed as illustrated in Fig 2 B The selected gracile and cuneate areas will be denoted as GFA (gracile fasciculus area) and CFA (cuneate fasciculus area) respectively in the following These areas should according to the somatotopical organization, contain fibres from the sacral and lower lumbar segments in case of the GFA and fibres from the upper thoracic and lower cervical segments in case of the CFA (Ferraro and Barrera 1935 Walker and Weaver 1942, Glees *et al* 1951 Glees and Soler 1951 Kruger *et al* 1961)

Photographs were also taken of the dorsal columns in the L7 segment where the region chosen for study was located somewhat lateral to the median plane in the most dorsal part (see Fig 2 C) This area will be denoted as the LA (lumbar area) in the following The somewhat lateral location was chosen in order to avoid the field of Flechsig containing a bundle of thin descending fibres (see Bumke and Foerster 1936, Milart 1966) According to existing reports the fibres measured in the seventh lumbar segment would to some extent correspond to those in the GFA (Walker and Weaver 1942, Glees *et al* 1951) By observations in the first cervical segment of two kittens in which the left L6 L7 and S1 dorsal roots had been severed at the age of one and two weeks respectively 5 days before perfusion, it was confirmed that the left GFA contained degenerating fibres.

2. The lateral funiculus

Two neighbouring regions were investigated They were located in the most superficial part of the lateral funiculus in the first cervical segment slightly anterior and posterior respectively to a line running from the central canal

perpendicularly to the median plane (see Fig 2 A) It has been documented that fibres of the ventral and dorsal spinocerebellar pathways will be found within these regions (Grant 1962) Fibres belonging to the rostral spinocerebellar tract may be intermingled with the ventral spinocerebellar fibres (Oscarsson and Uddenberg 1964) Reports describing a somatotopical localization within the dorsal (Sherrington and Laslett 1903) and ventral (Yoss 1953) spinocerebellar tracts in the dog and monkey respectively indicate that the spinocerebellar fibres in the regions selected for the present study originate in the caudal part of the spinal cord The anterior and posterior regions will be called ALF (anterior lateral funiculus) and PLF (posterior lateral funiculus) respectively

3 The pyramid

In a study where many animals of different ages are to be used all fibres of the pyramids cannot be measured since the feline pyramid contains at least 186,000 fibres (Lassek and Rasmussen 1940) A somatotopical localization within the corticospinal tract has been demonstrated above the level of the pyramid (see Peele 1961) but such an organization is not apparent in the pyramid at least not in monkeys (Coxe and Landau 1970) On the contrary pyramidal fibres originating from different cortical motor areas are homogeneously distributed within the pyramid and regional variations in fibre size distribution are minimal (Lassek and Rasmussen 1940 Brookhart and Morris 1948 Hess 1954 Breazile *et al* 1967) It was then decided to measure the fibres within the ventral part of the pyramid (see Fig 2 D) since this is an easily recognizable area and contamination with other fibre systems, such as the medial lemniscus, is avoided. On the basis of the preceding discussion it is thought that the results from this restricted area may be representative for the pyramid as a whole (cf Hess 1954)

Procedure of measurements

From each area one or two fields were photographed. The linear magnification on the paper copies was 1000 times. The measurements were performed with a Zeiss TGZ-3 particle size analyzer according to the method of Romero and Skoglund (1965) The same method has been used in a few earlier reports on developing central fibres (Bernstein 1966 Fleischhauer and Wartenberg 1967) All measurements were made by the same person and during one time period. The diameters measured included the myelin sheaths. Certain rules were followed in case of fibres which did not have an approximately circular outline. Oval fibres were measured over the shortest diameter pearshaped

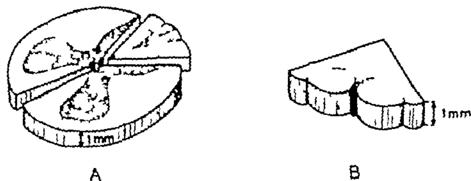


Fig. 1 Schematic drawings to show (A) how the transverse slices of the first cervical spinal cord segment were subdivided and (B) the appearance of the pyramidal specimens.



Fig. 2 Schematic drawings to show the localization of the different areas studied (A) ALF (1) and PLF (2) in the lateral funiculus of the first cervical segment. (B) GFA (2) and CFA (1) in the first cervical segment dorsal column. (C) LA in the seventh lumbar segment dorsal column. (D) The pyramidal area in the medulla above the decussation. Compare Fig. 1. Further explanation in the text

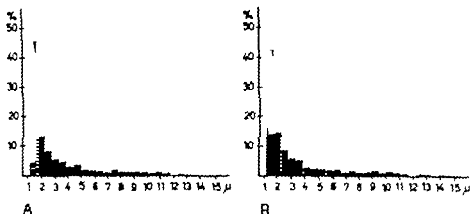


Fig. 3 (A and B) Histograms of calibre spectra of an adult ALF area to show the reproducibility of the measurements. The two calibre spectra were made from measurements on identical paper copies.

over the base and very irregular fibres were not measured (cf Schwieler 1968). In each defined area around 1000 fibres were usually measured. In the most immature cats the number of myelinated fibres did not always reach such values.

In older kittens and adult cats the myelinated fibres were so densely packed that the outer contour could not always be visualized *in toto* for each fibre. However it was nearly always possible to identify the outer contour at some points of the periphery of these fibres and thereby the myelin sheath thickness could be estimated and the appropriate fibre size determined. When the outer contour was completely obscured the myelin sheath thickness of similarly sized fibres, where the outer contour was clearly demarcated was adopted. As a source of error this will certainly be of little importance since the fibres were measured in classes with intervals of about 0.5 μ. It seems likely that the errors of measurements at the most might cause a shift from one size class to the next above or below. Since the aim of the present study is to describe a change in the calibre spectrum during postnatal maturation, with particular reference to the largest fibres rather than the absolute fibre sizes at each stage this error will have a negligible influence on the results. For technical reasons fibres below the size of 1.20 μ are not included in the measurements. Thus the proportion of very small myelinated fibres will be underestimated since central fibres myelinate at about 0.5 μ (Gaze and Peters 1961, Fleischhauer and Wartenberg 1967, Matthews 1968). The measuring of oval fibres over the smallest diameter and pearshaped over the base might cause a slight shift to the left of the fibre size distribution. In order to check the reliability of the measurements the fibre sizes in an ALF area of an adult cat were independently determined upon two identical paper copies. As illustrated by the histograms A and B in Fig 3 the two measurements gave similar results.

Methodological considerations

1 Fixation

Most reports on the fibre size distribution of developing and adult central fibre tracts are based on material fixed by immersion. Among the few exceptions the studies of the developing feline corpus callosum by Fleischhauer and Wartenberg (1967) and of the adult rat optic nerve by Forrester and Peters (1967) may be mentioned. It seems likely that different fixation methods will be reflected in the calibre spectra. In particular the large central fibres appear badly preserved with irregular contours and disrupted myelin sheaths after immersion fixation (see Luse 1960, Condie *et al* 1961). Another factor of

importance is the fixative itself. Rexed (1944) found that fixation of peripheral nerves in 10 % formaldehyde did not cause any size change whereas lower concentrations caused swelling and higher resulted in shrinkage of the fibres. Osmium impregnation did not alter the fibre size. Most calibre spectrum measurements on developing peripheral nerves which are of interest have been performed on nerves fixed by osmium tetroxide without further staining. This results in a distinct contour of large cross sectioned fibres but small fibres are less distinct and easily overlooked (see Rexed 1944, Schwieler 1968, Berthold and Carlstedt, to be published). As for the central pathways various fixatives have been employed and only a few studies are based on glutaraldehyde fixed postosmicated material (Fleischhauer and Wartenberg 1967, Samorajski and Friede 1968). In the case of central nervous tissue the vehicular tonicity is particularly critical since hypotonic and, with regard to immersion fixation, even normotonic vehicles cause a glial swelling (Zadunaisky et al. 1963, Schultz and Karlsson 1965, Torack et al. 1966) which leaves less space for the fibres.

2. Dehydration and embedding

According to Williams and Wendell-Smith (1960) osmium fixation of peripheral nerve followed by embedding in paraffin caused a 20 % reduction in myelin sheath thickness whereas the fibre diameter did not change appreciably (cf. Rexed 1944). Similarly osmium fixation and plastic embedding reduces the peripheral myelin period 30 % (Finean 1961, Sjöstrand 1963). A shrinkage of the same order was noted after glutaraldehyde fixation and Vestopal embedding (Karlsson 1966). Hursh (1939) found a very slight diameter increase of feline spinal root bundles during osmium fixation and after dehydration the bundle diameter had undergone a 24 % reduction in diameter. A considerable part of this shrinkage was due to the connective tissue whereas the individual fibre diameters decreased approximately 10 % during dehydration. There were no indications of a differential shrinkage in fibres of different sizes.

Mellström and Skoglund (1969) pointed out that the spinal cord shrinkage caused by fixation and embedding procedures is not uniform during postnatal development, the shrinkage being greater in the most immature animals. However, all tissue components within one specimen do not behave uniformly. As stated by Lodin et al. (1969) the greatest changes take place in the glial cells. Thus the myelinated fibres are not necessarily as severely affected. This might explain the finding of Mellström and Skoglund (1969) since the number of glial cells per unit area of a section is much higher in immature animals than at more mature stages (Roback and Scherer 1935, Hillebrand 1966).

3 The use of semithin toluidine blue stained Vestopal sections

As stated by Schwieler (1968) working with peripheral nerves, the use of semithin (0.5—1.0 μ) stained sections improves the quality substantially in comparison with paraffin sections. In particular the small fibres become more clearly visible (Berthold and Carlstedt to be published) A comparison of the fibre counts in the immature feline sural nerve by Ekholm (1967) who used an osmium-paraffin method and Schwieler (1968) who used glutaraldehyde followed by buffered osmium as fixative Vestopal as embedding medium and toluidine blue staining, reveals that with the former method a total of about 250 fibres were seen in the neonatal stage whereas the latter method visualized over 600 fibres. The influence of the staining method upon the results was also demonstrated by Brookhart and Morris (1948) who found obvious differences between the calibre spectra of osmium and silver stained specimens from the feline pyramid.

Abbreviations used in the text

LA An area in the most dorsal part of the dorsal column in the seventh lumbar spinal cord segment somewhat lateral to the median plane (Fig 2 C)

GFA An area in the most dorsomedial part of the gracile fasciculus in the first cervical spinal cord segment (Fig 2 B)

CFA An area in the most dorsomedial part of the cuneate fasciculus in the first cervical spinal cord segment (Fig 2 B)

ALF An area in the superficial part of the lateral funiculus in the first cervical spinal cord segment slightly anterior to a line drawn perpendicularly to the median plane from the central canal (Fig 2 A)

PLF An area in the superficial part of the lateral funiculus in the first cervical spinal cord segment slightly posterior to a line drawn perpendicularly to the median plane from the central canal (Fig 2 A)

PYR An area in the ventral part of the pyramid above the level of the decussation (Fig 2 D)

Results

A. General development

The general appearance of the areas of white matter which were investigated (except ALF) is illustrated in Fig 4 The ALF was omitted since its development in all respects is similar to that of PLF The pictures have been arranged

in order to enable a comparison of the lumbar dorsal column area (LA) with the cervical gracile fasciculus area (GFA). These two areas should contain the initial and terminal parts of the same dorsal column fibres (Glees *et al.* 1951). The GFA can in its turn be directly compared with the cuneate fasciculus area (CFA), the fibres of which have a more rostral origin (Glees *et al.* 1951). These three areas containing afferent fibres of dorsal root origin can then be compared with the PLF. Within this area dorsal spinocerebellar fibres originating from Clarke's column neurons in the lumbar spinal cord should be found (Sherrington and Laslett 1903, Grant 1962). Finally the general development of these afferent fibre systems can be compared with that of an efferent tract—the corticospinal pathway in the pyramid.

In the neonatal stage the LA, CFA and PLF are ahead of the GFA and PYR with respect to the number of myelinated fibres present (Fig. 4, first column). It should be noted that the last picture in the first column, showing the pyramidal area, was taken from a one week old kitten whereas the other pictures in this column refer to one day old animals. In the LA and CFA many small myelinated fibres are present together with several glial cells. Among the latter the characteristic myelinating oligodendrocytes were common (Bunge *et al.* 1962, Peters 1964). The PLF contained fewer myelinated fibres although some of them were as large as the largest LA and CFA fibres. The GFA showed very few thinly myelinated small fibres and most of the cross-sectional area was occupied by glial cells and what appeared as bundles of unmyelinated fibres. In the newborn pyramid myelin sheaths were absent (Purpura *et al.* 1964).

During the first and second postnatal weeks the number and size of the myelinated fibres increased. In the pyramid myelination had started in one week old animals but was limited to very few fibres (last picture in first column).

After three weeks (Fig. 4, second column) the LA and PLF are obviously much ahead of the other areas with respect to fibre size, showing some fairly large and heavily myelinated fibres. The CFA is ahead of the GFA and both areas show many small homogeneously sized fibres. The pyramidal area contains a great number of very small myelinated fibres.

Later on the same trend was followed and at six weeks (Fig. 4, third column) the LA is ahead of all the other areas with respect to the number of large fibres. The PLF has some equally large and slightly larger fibres but in contrast to the LA small and very small fibres predominate. Both the CFA and GFA have a homogeneous population of rather small fibres. In the pyramidal area a few individuals now seem to grow faster than the bulk of fibres which remain very small.

In the adult the trend seen during development is retained (Fig. 4 last column) The LA has many large fibres and in the PLF equally large and larger fibres are present although less numerous. The gracile fibres are smaller than the cuneate and in both areas the fibre population has a more or less homogeneous appearance. The pyramid on the other hand shows a few scattered large fibres and most fibres remain small or very small.

It should be mentioned that during the first postnatal weeks certain irregularities in the immature myelin sheaths and a number of peculiar glial features were noted. These are described in a separate paper (Hildebrand to be published) and will not be considered here.

In general it thus appears that at all developmental stages the LA is ahead of the other areas with respect to the number of large fibres. The terminal extensions of the LA fibres in the gracile fasciculus are however slowly growing being surpassed by the CFA fibres which are of cervical or thoracic origin. In each of the dorsal column areas the fibre population appeared fairly homogeneous with regard to the sizes. In the PLF a few fibres, which grew as fast as or faster than those of the LA, were intermingled with a majority of small fibres. A similar pattern was found in the pyramid which aquiring its first myelin sheaths by the age of one week, showed a development which was much delayed compared to the afferent fibres.

B. The fibre size distribution

The results of the measurements are presented as calibre spectra in Tables I—VI and as histograms in Fig 5 No calibre spectra were made from the pyramidal area of the most immature animals since this area was unmyelinated or poorly myelinated before the end of the second week. The ALF is not presented in histograms since its change with development was similar to that of the PLF

In the newborn stage the lumbar dorsal column area (LA) showed the most advanced stage of development with regard to the fibre sizes. The maximal fibre size was 3—3.5 μ and about 60 % of the fibres belonged to the first size group The fibre size distribution of gracile (GFA) and cuneate (CFA) fibres did not differ much from each other in spite of the higher degree of myelination in the latter Here 70—75 % of the fibres fell into the first size group and the maximal fibre size of 2.5—3 μ was reached by very few fibres. In the PLF over 80 % measured close to 1 μ most of the remaining fibres measured 2 μ and very few fibres exceeded that size maximally reaching about 3 μ

During the first postnatal week, not illustrated by histograms, the LA fibres continued to grow whereas the other areas changed very little

At the end of the second week the largest LA fibres measure around 4.5 μ and the group ≥4 μ comprised 1—3 % of the fibres. In the GFA and CFA the first size group was reduced by nearly 10 % the fraction of fibres at 2—3 μ was larger—particularly in the CFA. A few cuneate fibres reached over 4 μ. The PLF showed a moderate increase in 2—3 μ fibres and the maximal fibre size was as in the GFA, close to 4 μ. Nearly 90 % of the pyramidal fibres belonged to the first size group and very few fibres reached the maximal fibre size at about 3 μ.

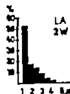
By the end of the third week the comparatively rapid growth of the LA fibres had increased the fibres ≥ 4 μ to 6 % and the first size group was reduced to 40 %. The largest fibres now measure over 5 μ. The GFA and CFA showed less conspicuous changes. As in the LA the PLF maximal fibre size had increased to over 5 μ but most fibres (70 %) remained close to 1 μ. Thus at this stage the largest PLF fibres had surpassed those of the GFA and CFA. The pyramid showed less clearcut differences between two and three weeks old animals, as seen from Table VI.

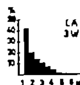
At an age of 5—6 weeks the LA, GFA and CFA maximal fibre size had increased to 7—8, 4—5 and 5—7 μ respectively. 20 % of the LA fibres but only 1 % of the GFA fibres reached a size of 4 μ or more. The CFA occupied an intermediate position. Both the GFA and CFA showed a distinct increase in the proportion of fibres at around 3 μ. In the PLF the largest fibres had increased in size to 7—9 μ which exceeds the size of the largest LA fibres. However as many as 60 % remain close to 1 μ and the proportion of fibres with a diameter of 4 μ or more comprised 6—14 % which is less than in the LA. The pyramidal area showed a fibre size distribution similar to that of the three week old PLF with 60—70 % in the first size class and a maximal fibre size around 5 μ.

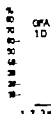
By three months the first size group had become reduced to about 30 % in all the dorsal column areas. 24 % of the LA fibres measured 4 μ or more and the largest of these were about 11 μ. In the gracile fasciculus the maximal fibre size was 6—7 μ. 6—14 % measured 4 μ or more and a peak had appeared at 3—4 μ. The largest cuneate fibres had grown to 8—9 μ and 15—25 % of the fibre population was 4 μ or more. In the PLF the largest fibres reach 12—14 μ. 45 % remain close to 1 μ and the proportion of fibres ≥ 4 μ had increased to 8—19 %. The pyramidal area showed an increase in the group of fibres measuring 4 μ or more to 5 % and a few fibres reached a size of about 8 μ. Thus at this stage the pyramid has larger fibres than the GFA.

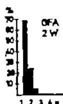
In the adult stage the LA showed a maximal fibre size of 12—15 μ and 30—45 % measured 4 μ or more. Still about 30 % remained close to 1 μ. The largest GFA fibres were 7—9 μ. 17 % measured 4 μ or more and the first size



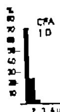


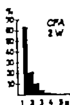


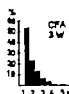


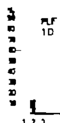














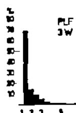
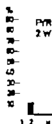
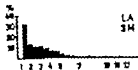


Fig 5 Histograms of calibre spectra of the investigated areas (except ALF) Ages and areas are indicated in the figur (D = days, W = weeks M = *months*) Further explanation in the text.

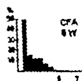





LA

LA
3 M

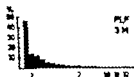
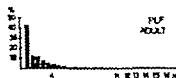
LA
ADULT

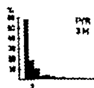

3 M

ADULT

CFA

CFA
3 M

CFA
ADULT

PLF

PLF
3 M

PLF
ADULT

PYR

PYR
3 M

PYR
ADULT

group comprised only 20 %. The peak at 3—4 μ was still present. In the cuneate area the largest fibres reached 9—11 μ. 20—30 % reached 4 μ or more and the first size group was as in the LA. Thus in the dorsal columns LA showed the largest fibres but also a high proportion of small fibres whereas the GFA possessed fewer fibres close to 1 μ, a peak at 3—4 μ and a smaller maximal fibre size. The CFA was intermediate between the two other dorsal column areas being more similar to the LA. In the adult PLF a few fibres measured 15—16 μ. The first size group was unchanged and 13—22 % measured 4 μ or more. In the pyramid a large proportion (50—60 %) of fibres remain close to 1 μ and only 6—9 % reached 4 μ or more. The largest pyramidal fibres had surpassed the largest fibres of both the GFA and the CFA being of a size approaching that of the large LA fibres (12—14 μ).

In Fig. 6 the size of the largest fibres in each of the areas studied (except ALF) has been plotted against age. In the dorsal columns the LA fibres show the most rapid growth followed by the CFA whereas the GFA fibres grew more slowly. In these three areas the growth was most conspicuous during the first six weeks, somewhat slower up to three months and thereafter went on at a slow rate. In the PLF the increase in maximal fibre size followed a slightly different pattern in that a high growth rate was maintained throughout the first three postnatal months. The pyramidal maximal fibre size increased during the first three months and continued to grow at a somewhat reduced rate up to the adult stage.

In Fig. 7 the percentage of fibres measuring 4 μ or more in each of the areas studied (except ALF) has been plotted against age. LA fibres of this size first appear during the second week and increase greatly in number up to six weeks whereafter the percentage continues to grow at a slower rate. In GFA such fibres do not appear until the fifth or sixth week and the percentage increases at a fairly even to the mature stage. In the cuneate area 4 μ fibres appear during the second and third weeks. Many new fibres are added to this group up to three months after which the percentage continues to increase more slowly. In the PLF fibres with a size of 4 μ or more are at hand at the end of the third week. This group expands rapidly during the following three weeks. Few fibres are added after the third month. The pyramidal area differs markedly from the other areas with respect to the number of fibres $\geq$ 4 μ. Appearing first at six weeks these fibres increase a few per cent up to three months and thereafter very small changes take place. This reflects the persistence of a high proportion of small fibres in the pyramid throughout development.

The measurements presented clearly show that at birth the LA has reached the most advanced stage of development followed by CFA and PLF. At the

end of the third week the largest fibres are seen in the LA and PLF whereafter PLF surpasses LA with respect to maximal fibre size. The largest pyramidal fibres, being unmyelinated during the first postnatal week, surpass the largest fibres of both the GFA and CFA during growth to the adult stage. It is also clear that in both PLF and PYR there is a small group of fibres which myelinate first and attain a large size in the mature stage whereas the bulk of fibres remain at a small size. In the dorsal column areas the percentage of fibres > 4 μ is high and the first size group comparatively small. This might be a reflection of the more homogeneous fibre content of the dorsal columns whereas in the PLF and PYR a few large spinocerebellar and corticospinal fibres are present together with a large population of small fibres the identity of which is unknown.

In general it seems that the fibre systems mature in a certain order First comes the LA and for a short period the CFA followed and later surpassed by PLF The GFA, containing the terminal extensions of the LA fibres, develops at a slower rate. At last when the development of the afferent systems has been going on for some time the myelination and subsequent maturation of the corticospinal system ensues.

Discussion

It should be pointed out that the fibre sizes reported in this study include the myelin sheaths. Consequently the size growth recorded is the sum of increasing myelin sheath thickness and growing axon diameter According to Samorajski and Friede (1968) the axons in the developing rat pyramidal tract might control the formation of their own myelin sheaths by calibre changes. In the developing guinea pig pyramidal tract Hess (1954) noted alternate periods of fibre size enlargement and waves of myelination. On the other hand Gaze and Peters (1961) suggested that the establishment of synaptic connections is a more important stimulus to myelination than calibre changes. Thus visual function promotes myelination of the optic nerve in developing mice (Gyllensten and Malmfors 1963) The importance of function as a stimulus to nervous maturation was also pointed out by Kingsley *et al.* (1970)

The dorsal columns

In the dorsal columns the dorsal root afferents concerned with tactile discrimination and kinaesthesis run cranialwards to terminate in the dorsal column nuclei (see Brown 1968 Jabbur and Banna 1968 Kitai and Weinberg 1968,

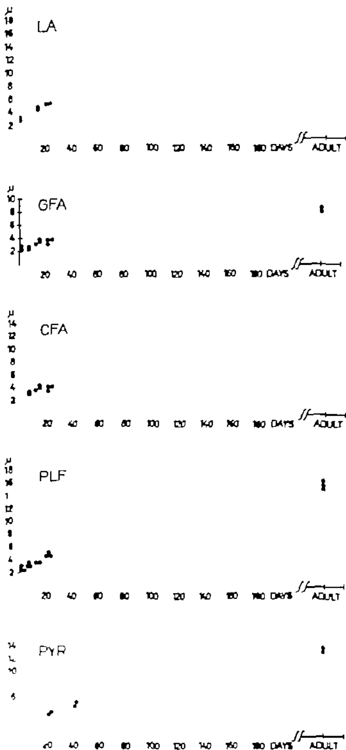


Fig. 6 Size of the largest fibres in the different areas (except ALF) plotted against age.

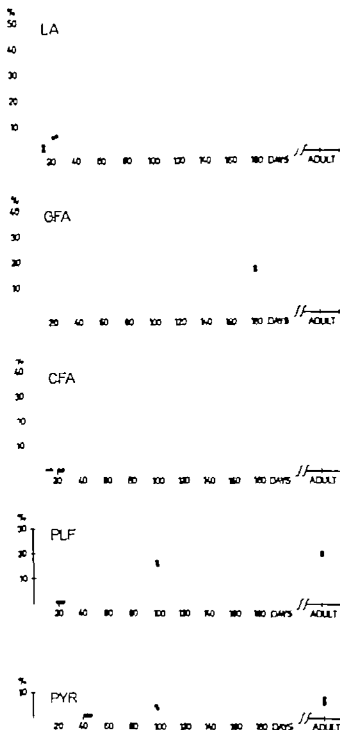


Fig. 7 The percentage in the investigated areas (except ALF) of fibres measuring 4 μ or more plotted against age.

Petit and Burgess 1968 Uddenberg 1968 Whitsel *et al.* 1969) The two dorsal column regions which were investigated in the first cervical segment (GFA, CFA) should contain afferents from the hind and fore-limb respectively (Glees and Soler 1951 Glees *et al* 1951)

The measurements clearly demonstrate that the gracile fasciculus develops later than the cuneate as also noted by earlier workers (Langworthy 1929 Windle *et al.* 1934) The adult maximal fibre size was 7—9 μ in the gracile and 9—11 μ in the cuneate area. Fibres measuring four microns or more first appear at 5—6 weeks in the gracile and at 2—3 weeks in the cuneate fasciculus It was furthermore found that in the adult a rather high proportion of fibres measured 4 μ or more whereas the very small fibres were less numerous than in the other fibre tracts investigated. In principle the findings in the adult stage agree with the measurements of Häggqvist (1936) and Szentágothai-Schimert (1941/42) in man. However the techniques used by them obviously obscured the smallest fibres. Therefore the percentage of large fibres given by them is probably too high.

In the dorsal funiculus area of the seventh lumbar segment (LA) the initial and in the gracile area the terminal part of the same fibres should be found (Glees *et al* 1951) However the lumbar area was more advanced with respect to fibre size and myelination than the gracile area at all stages of development. The largest adult LA fibres measured 12—15 μ and as in the gracile and cuneate fasciculi the number of fibres $\geqslant 4$ μ was comparatively large whereas the very small fibres were less numerous than for example in the pyramidal tract (cf Szentágothai-Schimert 1941/42)

It is likely that the lumbar dorsal column fibres measured here partly represent the central extensions of fibres in the upper sacral dorsal roots (Glees *et al* 1951) A growth pattern similar to that in the lumbar area was demonstrated in the first sacral dorsal root by Skoglund and Romero (1965)

Only 22—23 % of the dorsal column fibres reach the gracile and cuneate nuclei without interruption (Glees and Soler 1951) Whitsel *et al.* (1969) concluded that as the gracile fibres ascend from lumbar to cervical levels, fibres subserving deep sensibility leave the dorsal column (cf Brown 1968) It is clear from the studies of Burgess *et al.* (1968) and Petit and Burgess (1968) that afferent fibres with a higher conduction velocity notably the larger tend to project to the dorsal column nuclei to a greater extent than those with slower rates of conduction (cf. Wall 1961) Thus the difference in maximal fibre size at lumbar (LA) and cervical (GFA) levels cannot be explained only by disappearance of larger fibres between the two levels. Many of the large dorsal column fibres branch in the upper lumbar to lower thoracic cord and possibly also at higher levels (Gardner *et al.* 1949 Rexed and Strom 1952,

Wall 1961) If fibre size is reduced after branching (Lloyd and McIntyre 1950 Rexed and Strom 1952, Lehmann 1959) this may explain a decrease in size of the large lumbosacral dorsal column afferents as they approach cervical levels.

Bement and Ranck (1969) found few gracile fibres with a conduction velocity over 57 m/sec (cf Brown 1968) which corresponds to a fibre size of 10 μ (Hursh 1939) in the lower cervical and upper thoracic dorsal funiculus of the cat. The maximal conduction velocity recorded by them indicated a maximal fibre size of 11—12 μ which is lower than the maximal fibre size in the LA but higher than the largest GFA fibres. This fits well into the pattern of a decreasing size of dorsal column fibres as they ascend (see Bement and Ranck 1969)

Petit and Burgess (1968) pointed out that the conduction velocity decreased 35—70 % as sural nerve fibres entered the dorsal columns. Between the C2 and Th10 segments they recorded a maximal conduction velocity of 45 m/sec. The size of these fibres would be about 8 μ which fits well with the measurements presented here. Since sural nerve fibres enter the spinal cord via the seventh lumbar and first sacral dorsal roots (Ekholm 1967) the reduction in conduction velocity observed by Petit and Burgess (1968) would be explained by the decrease in fibre size The different reduction in conduction rate in fibres from different receptor types, observed by these authors (cf Brown 1968) could be explained by a varying degree of branching The statement that conduction velocity decreases as peripheral fibres enter the dorsal funiculus (see also Lloyd and McIntyre 1950 Collins and Randt 1961 Brown 1968) is supported by the observation of Maxwell *et al* (1969) and Wulfhekel (1969) that peripheral fibres entering the central nervous system decrease in diameter and myelin sheath thickness.

The evidence in the present report of an earlier development of hindlimb dorsal column afferents at lumbar than at cervical levels might seem to be in contrast to the generally accepted concept of a craniocaudal development of the nervous system (Langworthy 1929 Kingsbury 1932) and is in apparent disagreement with the statement of Fox *et al.* (1967) that in the developing canine spinal cord the funiculi of the cervical region appear more myelinated than those at lumbar levels. From a comparison between the development of the gracile and cuneate fasciculi it is clear that the forelimb dorsal column fibres are ahead of those of the hindlimb in good accordance with the concept. This does however not imply that myelination proceeds in a craniocaudal direction along each fibre tract. On the contrary the hindlimb dorsal column fibres develop in a caudocranial direction as documented in the present report. This seems quite reasonable since these fibres have their cell-bodies located at

lumbosacral levels (Glees and Soler 1951) A consequence of the statement by Fox *et al* (1967) would be that ascending fibres myelinate first at their terminal parts, which appears highly unlikely

On the basis of the present results and the discussion above it should be stated that the concept of craniocaudal development is valid only with respect to functional maturation but not with respect to the morphological maturation of the spinal cord funiculi or single fibres. The development of each fibre proceeds in a somatofugal direction from the cell-body towards its termination (cf Nyberg Hansen and Rinvik 1963) Thus ascending fibre tracts will develop and myelinate in a caudocranial direction. Within each ascending fibre tract the fibres concerned with cranial parts of the body will develop before those concerned with caudal parts, which is the morphological substrate for the craniocaudal development of function. In descending fibre tracts each fibre will myelinate in a somatofugal direction from the cranially situated cell body caudalwards. Descending fibres concerned with cranial structures will be ahead of those concerned with caudal parts of the body (Conel 1941 Humphrey 1960) However the results of Marty and Scherrer (1964) and Banik *et al* (1968) are in opposition to the discussion above indicating that in the kitten optic system myelination proceeds in two directions from the neighbourhood of the chiasma towards both the eyeball and the more central parts of the visual pathway

The lateral funiculus regions

Within the regions denoted as PLF and ALF fibres of the dorsal and ventral spinocerebellar pathways should be present (Sherrington and Laslett 1903 Laporte *et al.* 1956 a, b Laporte and Lundberg 1956, Grant 1962) It is well known that these two pathways are characterized by large fibres (Häggqvist 1936 Szentágothai-Schimert 1941/42) The dorsal spinocerebellar tract serves proprioceptive functions, transmitting impulses from ipsilateral trunk and hindlimb muscle afferents (muscle spindles, Golgi tendon organs) to the cerebellar cortex (Laporte *et al* 1956 a, b Laporte and Lundberg 1956) and via the ventral spinocerebellar pathway impulses from contralateral skin and joint afferents and high threshold muscle afferents pass to the cerebellum (Oscarsson 1957) The ventral tract originates in the dorsolateral portion of the ventral gray of mainly the fourth and fifth lumbar segments (Carrea and Grundfest 1954 Ha and Liu 1968) and the dorsal tract comes from columna Clarkii, which in the cat extends from L3-4 up to Th1 2 (Rexed 1954 Grant and Rexed 1958) The spinocerebellar fibres measured here probably come from the caudal part of the spinal cord (Sherrington and Laslett 1903 Yoss 1953)

In the first cervical segment the two regions analyzed (ALF PLF) do not differ in any clearcut way from each other with respect to the growth of fibre diameters. Both are partly myelinated at birth (cf Tilney and Casamajor 1924 Langworthy 1929) In the adult the largest fibres had a size of 14—17 μ which would correspond to a maximal conduction velocity of about 100 m/sec (Hursh 1939) The Hursh factor was established from studies on peripheral nerves (Hursh 1939) but seems to be valid also for central fibres (Bishop *et al* 1953 Bishop and Clare 1955 Chang 1956, Lennox 1958) A calculated maximal conduction velocity of 100 m/sec is in accordance with experimental findings (Lloyd and McIntyre 1950 Laporte *et al.* 1956 a, Oscarsson 1957)

Decerebrate rigidity of the hindlimbs is not present in kittens younger than three weeks (Skoglund 1960 a) This was found to be largely dependent on the immature stage of the afferent link from muscle spindles and lack of gamma support before that age At the age of three weeks the bulk of the peripheral 1 a afferents from the kitten hindlimb reach close to adult functional properties corresponding to a fibre size of about 4 μ and a conduction velocity of 18—20 m/sec (see Skoglund 1960 b Skoglund and Romero 1965) Some of these fibres form the first link in the pathway from muscle receptors to the cerebellum. The second link is furnished by the dorsal spinocerebellar fibres, the two links in the chain being synaptically joined in Clarke s column. Decerebrate rigidity "the exaggerated reflex of standing" is probably relayed over the cerebellum (Terzuolo and Terzian 1953) At the age of three weeks the largest ALF and PLF fibres, which at least partly represent spinocerebellar fibres (Grant 1962) have reached a size around 4 μ and the maximal fibre size is rapidly growing As discussed below in connection with the developing pyramidal tract a rapid increase in the conduction velocity of central fibres seems to be initiated at a fibre size around 4 μ It is then tempting to correlate the maturation of the spinocerebellar fibres to the postnatal appearance of decerebrate rigidity in the hindlimbs and to the physiological maturation of hindlimb muscle spindle afferents shown by Skoglund (see Skoglund 1966) Accordingly morphological studies indicate that with respect to the dendritic organization the neurons of Clarke s column and to some extent also the cerebellar Purkinje cells present a basically mature pattern in three week old kittens (Purpura *et al.* 1964 Smith 1969)

The pyramid

In the feline pyramid myelinated fibres were not present at birth (cf Tilney and Casamajor 1924 Langworthy 1929 Purpura *et al* 1964) In the adult the maximal fibre size was 12—14 μ (cf Brookhart and Morris 1948 Purpura

et al 1964) In the pyramid as well as in all other central fibre tracts there is a predominance of small fibres (Häggqvist 1936, 1937 Lassek and Rasmussen 1940 Lassek 1942 a, Brookhart and Morris 1948 Purpura *et al.* 1964) In the present material only 6—9 % of the adult pyramidal fibres measured 4 μ or more

The postnatal development of human pyramidal fibres is rather protracted as pointed out by Lassek (1942 b) This is in accordance with the slow development found in the kitten (cf Bernstein 1966)

The functional maturation of the feline pyramidal tract has been investigated by Purpura *et al* (1964) and Huttenlocher (1970) The former authors reported that the conduction velocity of the pyramidal fibres giving rise to the shortest latency response increased very slowly up to the age of three weeks. The velocities measured ranged from 0.8 to 2.0 m/sec. Since myelination was well under way at the age of three weeks the initial deposition of myelin seemed not to affect the conduction velocity particularly much (see Discussion in Marty and Scherrer 1964) During the fourth and fifth weeks the conduction velocity increased rapidly and then continued to increase but at a progressively slower rate until the adult stage. The maximal adult conduction velocity was about 90 m/sec. During the critical period at 4—5 weeks conduction velocities of 12—20 m/sec were recorded. According to Huttenlocher (1970) feline pyramidal tract fibres are not able to fire repetitive impulses at high stimulus rates for any longer periods until between the third and fifth postnatal weeks. At the end of the first month the dendritic pattern of the large pyramidal neurons appeared mature and the pyramidal tract was electrographically similar to that in the adult stage (Purpura *et al* 1964) Since the pyramidal maximal fibre size at the age of 2—3 weeks is around 3 μ and has reached 5 μ at 6 weeks the great change in conduction velocity reported by Purpura *et al.* (1964) and the acquisition of ability to maintain sustained activity (Huttenlocher 1970) occurs at a fibre size between three and five microns. If the corrected Hursh factor (Hursh 1939 Ekholm 1967) is applied to the conduction velocities of 12—20 m/sec, which were found at the end of the first postnatal month a theoretical fibre size of 3—4 μ is obtained. From the adult maximal conduction velocity of 90 m/sec (Purpura *et al.* 1964) a theoretical maximal fibre size about 15 μ can be calculated. Obviously the calculated fibre sizes are in accordance with the present results. The figures calculated also fit remarkably well with those of developing peripheral fibres, which in several respects appear physiologically and morphologically mature at a conduction velocity of 18—20 m/sec corresponding to a diameter around 4 microns (see Skoglund 1966 and Berthold and Skoglund 1965 1967 1968 a, b, Berthold 1968 a)

Generally the measurements presented here and the discussion above suggest that in developing central fibres the correlation between fibre size and function is similar to that in developing peripheral nerve fibres. It also appears that, with respect to the central fibres studied, maturation (myelination) proceeds in a somatofugal direction from the cell-body towards the termination and that the afferent fibre tracts investigated develop before the efferent corticospinal pathway. This supports the idea (see Nyberg Hansen and Rinvik 1963) that in a chain of coupled neurons maturation propagates in the same directions as the conduction of impulses.

Summary

The postnatal development of the fibre composition (calibre spectra) in six defined areas in feline spinal cord and brainstem white matter was studied. The animals were fixed by glutaraldehyde perfusion and after post-osmication the pieces of white matter were embedded in Vestopal. For the measurements semithin (0.5—1.0 μ) toluidine blue stained sections were used. The studied areas were located in (1) the dorsal funiculus in the seventh lumbar spinal cord segment, (2) the gracile and cuneate fasciculi in the first cervical segment, (3) the superficial part of the first cervical segment lateral funiculus corresponding to the location of the ventral and dorsal spinocerebellar pathways and (4) the pyramid.

At all developmental stages small fibres predominate in the investigated areas. This is less pronounced in the dorsal column areas than in the lateral funiculus area but is very marked in the pyramid. The afferent tracts investigated were found to develop before the efferent corticospinal tract. In the dorsal columns the lumbar area is ahead of the cervical gracile and cuneate areas with respect to myelination and growth in fibre size at all stages of development. The findings are discussed in the light of studies of developing peripheral nerves and compared with physiological data. Generally the correlation between fibre size and function appears to be similar to that in developing peripheral nerves.

Acknowledgements

This investigation was supported by grants from Karolinska Institutet (Reservationsanslaget) and from the Swedish Medical Research Council (projects no. B70-14X-136-06 and B71 14X-136-07A). We are indebted to Mrs. Ingegerd Lindqvist and Miss M. J. Berghman for skilful technical assistance.

TABLE I Calibre spectrum of the LA.

Age in days	Animal nr	1.21–1.76	1.76–2.32	2.32–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94
1	K 11	447	194	45	5										
1	K 12	602	278	113	18										
1	K 13	649	335	137	8										
7	K 21	472	242	137	59	7	2								
14	K 23	460	198	133	94	57	9	1							
14	K 24	613	203	164	103	55	29	2							
21	K 9	419	211	172	134	80	49	15	3						
23	K 4	445	213	150	113	74	47	16	7						
35	K 27	491	206	156	131	107	100	81	70	42	13	6	3		
48	K 26	356	197	139	134	94	86	52	39	22	10	4	1	3	
99	K 16	310	129	106	108	85	65	53	33	21	15	8	4	8	6
174	K 31	375	162	125	125	80	67	40	33	30	20	13	11	9	5
180	K 33	346	137	108	110	83	67	65	55	53	38	24	29	15	9
adult	K 18	136	35	45	37	30	23	16	23	21	30	19	16	21	16
adult	K 56	206	139	97	68	52	39	33	27	14	15	13	7	6	7

TABLE II Calibre spectrum of the GFA.

Age in days	Animal nr	1.21–1.76	1.76–2.32	2.32–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94
1	K 11	115	37	5											
1	K 12	472	135	5											
1	K 13	189	51	3											
7	K 20	433	184	7											
7	K 21	297	99	4											
12	K 3	683	305	67	3										
14	K 23	452	195	69	11	3									
14	K 24	694	249	56	6	1									
1	K 9	533	223	53	3										
21	K 8	516	231	58	7										
23	K 4	574	234	95	13	1									
35	K 27	439	240	208	129	51	9	1							
43	K 14	420	293	242	128	33	6	3							
48	K 26	503	242	188	85	17	2								
90	K 15	288	146	201	206	151	96	38	6	3					
99	K 17	251	162	180	164	84	36	15	4	1					
99	K 16	201	97	131	117	128	57	34	13	3	3	1			
174	K 31	222	140	151	139	130	106	39	19	2					
180	K 33	255	168	203	184	147	109	32	20	11	3	3	1		
adult	K 28	271	178	197	187	125	80	20	5	3	1	1			
adult	K 18	268	169	187	176	173	138	79	39	17	13	8	9	4	
adult	K 58	112	61	81	72	60	51	47	30	17	15	13	8	4	1

10.04—10.60	10.60—11.15	11.15—11.70	11.70—12.25	12.25—12.80	12.80—13.36	13.36—13.91	13.91—14.46	14.46—15.01	15.01—15.56	15.56—16.12	16.12—16.67	16.67—17.22	17.22—17.77	Number of fibres measured
														687
														1011
														1129
														939
														932
														1169
														1083
														1065
														1406
														1137
3		2												967
	1		2											1101
4	2	5		1										1146
7	8	8	7	2	2									527
6	8	7	12	2	2	4	2	2	1					791

10.04—10.60	10.60—11.15	11.15—11.70	11.70—12.25	12.25—12.80	12.80—13.36	13.36—13.91	13.91—14.46	14.46—15.01	15.01—15.56	15.56—16.12	16.12—16.67	16.67—17.22	17.22—17.77	Number of fibres measured
														157
														612
														243
														624
														400
														1058
														730
														917
														812
														814
														1006
														1077
														1125
														1039
														1135
														897
														785
														948
														1166
														1068
														1280
														574

TABLE III. Calibre spectrum of th CFA.

Age in days	Animal nr	1.21–1.76	1.76–2.32	2.32–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94
1	K 11	714	230	28											
1	K 12	634	214	35	1										
1	K 13	643	140	22	1										
7	K 20	522	164	28	6										
7	K 21	454	144	77	16										
12	K 3	891	156	36	5	5									
14	K 23	519	155	75	36	12	3								
14	K 24	437	191	109	52	28	2								
21	K 9	613	226	106	51	9									
21	K 8	475	200	88	48	16	2								
23	K 4	630	215	103	39	14	1								
35	K 27	475	141	113	115	65	35	8	1	3					
43	K 14	391	184	159	126	88	53	36	29	16	12	3	2		
48	K 26	398	199	137	122	55	19	5	5						
90	K 15	341	148	153	151	97	86	46	22	17	8	13	4		2
99	K 17	298	150	133	117	122	77	62	40	40	17	15	2	4	4
99	K 16	347	151	152	109	93	61	34	23	10	5	2	5	6	
174	K 31	300	137	112	96	82	84	76	31	25	13	9	4	4	2
180	K 33	311	146	128	134	114	123	75	62	30	31	18	18	6	7
adult	K 28	320	157	144	119	102	83	59	23	15	14	8	17	4	
dult	K 18	316	133	160	135	100	76	62	60	35	21	21	17	13	11
adult	K 58	208	136	99	74	55	45	38	21	16	13	13	9	9	5

ABLE IV Calibre spectrum of the PLF

Age in days	Animal nr	1.21–1.76	1.76–2.32	2.32–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	9
1	K 12	567	33	4												
1	K 13	805	70	8												
1	K 10	709	108	8	1											
7	K 7	802	115	41	1											
7	K 22	668	69	32	7											
7	K 20	668	134	72	15	6										
12	K 3	789	119	64	27	13										
13	K 5	514	121	34	4	1										
14	K 24	384	97	52	11	2										
20	K 2	887	151	94	66	21	11	2								
21	K 8	800	163	101	52	21	16	2	3							
23	K 4	476	129	75	44	14	5	1								
35	K 27	491	134	105	74	46	22	25	10	3	1					
43	K 14	647	140	72	54	27	22	20	18	5	4	12	5	6	2	4
46	K 26	421	134	129	121	101	53	36	32	13	11	3				
90	K 15	575	167	141	97	63	48	35	31	20	15	17	9	11	5	2
99	K 16	453	152	114	74	48	37	21	19	21	14	7	8	7	9	7
99	K 17	490	160	153	96	55	46	22	20	16	18	10	21	10	2	3
adult	K 67	645	196	134	76	51	38	23	21	17	16	9	5	6	9	4
adult	K 65	662	183	176	118	87	60	45	25	18	27	24	20	10	13	15
dult	K 58	673	211	148	93	72	41	31	30	22	23	13	14	16	17	24

10.04–10.60	10.60–11.15	11.15–11.70	11.70–12.25	12.25–12.80	12.80–13.36	13.36–13.91	13.91–14.46	14.46–15.01	15.01–15.56	15.56–16.12	16.12–16.67	16.67–17.22	17.22–17.77	Number of fibres measured
														972
														881
														806
														720
														691
														1093
														800
														819
														1003
														829
														1000
														934
														1090
														940
														1068
														1003
														1003
														976
1	1													1210
														1066
6	2													1181
1	1	1												753

10.04–10.60	10.60–11.15	11.15–11.70	11.70–12.25	12.25–12.80	12.80–13.36	13.36–13.91	13.91–14.46	14.46–15.01	15.01–15.56	15.56–16.12	16.12–16.67	16.67–17.22	17.22–17.77	Number of fibres measured
														624
														883
														826
														939
														776
														893
														1012
														674
														546
														1252
														1158
														744
														911
														1038
														1054
			1											1237
2	2	1	1	1										1003
4	2		2		1		1							1139
3	2	7	3	3	3	4	1	1	1	1				1280
8	8	6	5	3	4	1	2	1	2	1				1535
17	12	6	12	1	1	1			1		1			1489

TABLE V Calibre spectrum of the ALF

Age in days	Animal nr	1.21–1.76	1.76–2.32	2.32–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	[illegible]
1	K 12	777	80	13												
1	K 13	875	74	17	2											
1	K 10	876	110	13	1											
7	K 7	921	157	49	12	2										
7	K 22	784	117	62	15	2										
7	K 20	681	113	69	19	2										
12	K 3	933	170	77	26	5										
13	K 5	558	129	33	9											
14	K 24	378	109	38	13											
20	K 2	959	154	74	41	16	3	3								
21	K 8	736	141	68	38	11	8	2	3							
23	K 4	633	137	123	48	14	5									
35	K 27	585	166	97	60	35	27	12	6	6	4	2	1			
43	K 14	700	143	92	66	49	24	23	11	8	7	9	5	3		
46	K 26	634	184	104	76	27	20	18	11	8	4	2	2		1	
90	K 15	428	150	97	86	60	36	32	23	18	18	13	15	17	11	
99	K 16	537	190	104	55	28	19	10	11	10	10	7	5	5	2	
99	K 17	526	130	96	66	49	38	31	18	14	17	17	18	21	10	
adult	K 67	913	272	212	101	62	50	28	23	21	19	14	17	9	12	
dult	K 65	780	203	149	119	78	64	59	38	21	31	18	19	21	15	1
adult	K 58	797	210	144	104	73	59	39	33	18	18	20	20	12	8	1

ABLE VI Calibre spectrum of the PYR.

Age in days	Animal nr	1.2–1.76	1.76–2.32	2.32–2.87	2.87–3.42	3.42–3.97	3.97–4.52	4.52–5.08	5.08–5.63	5.63–6.18	6.18–6.73	6.73–7.28	7.28–7.84	7.84–8.39	8.39–8.94	[illegible]
12	K 3	293	38	1												
14	K 23	448	226	28	2											
20	K 2	695	117	23												
21	K 8	449	132	39	6											
23	K 4	687	136	29	9	1										
42	K 5	783	190	93	35	19	1	1								
43	K 14	793	271	98	43	20	15	2	2							
46	K 26	520	134	59	33	6	3	1								
90	K 15	733	[illegible]39	127	37	44	25	14	16	4	2	3	3			
99	K 17	736	256	140	60	43	14	10	12	4	6	3	1	1		
99	K 16	671	264	130	61	32	21	13	10	7	5	2	2	3		1
150	K 32	695	219	80	38	20	20	17	6	3	6	5	1	2		
174	K 31	706	286	143	51	31	16	13	15	7	6	3	4	2	1	
180	K 33	553	201	112	48	45	22	11	15	8	7	5	3	4	2	2
adult	K 34	854	399	246	95	49	33	30	21	25	8	8	13	8	6	4
adult	K 58	704	235	90	36	17	9	14	9	7	11	7	3	3	3	1
adult	K 115	822	314	142	60	37	20	20	14	20	9	4	2	3	3	2

10.04—10.60	10.60—11.15	11.15—11.70	11.70—12.25	12.25—12.80	12.80—13.36	13.36—13.91	13.91—14.46	14.46—15.01	15.01—15.56	15.56—16.12	16.12—16.67	16.67—17.22	17.22—17.77	Number of fibres measured
														670
														968
														1000
														1141
														980
														851
														1201
														729
														560
														1250
														1009
														960
														1001
														1140
														1092
4				1										1021
	1					1								1000
1	3	2		2	1									1071
6	3	2	3			1								1778
12	11	8	5	11	10	5	2		2					1706
7	12	11	10	12	7	5	4	5	5	4	1	2		1600

10.04—10.60	10.60—11.15	11.15—11.70	11.70—12.25	12.25—12.80	12.80—13.36	13.36—13.91	13.91—14.46	14.46—15.01	15.01—15.56	15.56—16.12	16.12—16.67	16.67—17.22	17.22—17.77	Number of fibres measured
														332
														701
														833
														626
														1062
														1122
														1244
														756
														1247
														1286
														1222
														1112
		1												1266
1			1											1040
2		1	1											1905
3	1		1	1	1									1157
1		1				1								1478

References

Banik, N L., Blunt M. J and Davison A. N Changes in the osmiophilia of myelin and lipid content in the kitten optic nerve. *J Neurochem.* *15* 471—475 (1968)

Bement S L. and Ranck, J B. A quantitative study of electrical stimulation of central myelinated fibres. *Exp Neurol.* *24* 147—170 (1969)

Bergland R. M. Newer concepts of myelin formation correlated to functional changes. *4 A. New ol. 2* 60—265 (1960)

Bernstein J J Relationship of corticospinal tract growth to age and bodyweight in the rat. *J Comp Neurol.* *127* 207—216 (1966)

Berthold, C H. Ultrastructure of postnatally developing peripheral nodes of Ranvier *Acta Soc Med Upsal.* *73* 145—168 (1968)

Berthold, C. H. A study on the fixation of large mature feline myelinated ventral lumbar spinal-root fibres. *Acta Soc Med Upsal.* *73* Suppl. 9 1—36 (1968 b)

Berthold C. H. and Skoglund S Ultrastructure and histochemistry of the developing node of Ranvier in the hindlimb nerves of the cat. *Acta Soc Med Upsal.* *70* 287—293 (1965)

Berthold C. H. and Skoglund S. Histochemical and ultrastructural demonstration of mitochondria in the paranodal region of developing feline spinal roots and nerves. *Acta Soc Med Upsal.* *72* 37—70 (1967)

Berthold C. H. and Skoglund S Postnatal development of feline paranodal myelin sheath segments. I. Light microscopy *Acta Soc Med Upsal.* *73* 113—126 (1968)

Berthold, C. H. and Skoglund, S Postnatal development of feline paranodal myelin-sheath segments. II. Electron microscopy *Acta Soc Med Upsal.* *73* 127—144 (1968 b)

Bishop G H. and Clark M. H. Organization and distribution of fibres in the optic tract of the cat. *J Comp Neurol.* *103* 269—304 (1955)

Bishop P O Jeremy D and Lance J W The optic nerve Properties of a central tract. *J Physiol* (Lond.) *121* 415—432 (1953)

Breazile, J E., Jennings, D P and Swafford B. C. Conduction velocities in the corticospinal tract of the horse. *Exp Neurol.* *17* 357—363 (1967)

Brookhart J M. and Morris, R. E. Antidromic potential recordings from the bulbar pyramid of the cat *J Neurophysiol.* *11* 387—398 (1948)

Brown A. G Cutaneous afferent fibre collaterals in the dorsal columns of the cat. *Exp Brain Res* *5* 293—305 (1968)

Bumke, O and Foerster, O *Handbuch der Neurologie* 5 Springer Berlin (1936)

Bunge, M. B., Bunge, R. P and Pappas, G. D Electron microscopic demonstration of connections between glia and myelin sheaths in the developing mammalian central nervous system *J Cell Biol* *12* 448—453 (1962)

Burgess, P R Petit D and Warren, R. M. Receptor types in cat hairy skin supplied by myelinated fibres. *J Neurophysiol.* *31* 833—848 (1968)

Carpenter, F G and Bergland, R M. Excitation and conduction in immature nerve fibres of the developing chick. *Amer J Physiol.* *190* 371—376 (1957)

Carrea, R M and Grundfest H. Electrophysiological studies of cerebellar inflow I Origin, conduction and termination of ventral spinocerebellar tract in monkey and cat. *J Neurophysiol* *17* 208—238 (1954)

Chang H T Fibre groups in primary optic pathway of cat. *J Neurophysiol.* *19* 224—231 (1956)

COLLINS, W F and RANDT C. T Fiber size and organization of afferent pathways. *A ch. Neurol. 5* 202—209 (1961)

CONDIE, R. M., HOWELL, A. E. and GOOD, R. A. Studies on the problem of preservation of myelin sheath ultrastructure: evaluation of fixation, dehydration and embedding techniques. *J Biophys. Biochem. Cytol. 9* 429—443 (1961)

CONEL, L. J Development of the human cerebral cortex during the first month of life. *A ch Neurol. Psychiat 45* 387—389 (1941)

CONW Y O. J W ight F S and BRADLEY W E. Electrophysiological maturation of the pyramidal tract in the postnatal rabbit. *Electroenceph. Clin. Neurophysiol 26* 565—577 (1969)

COOK, W S and LANDAU W M P tterns of Marchi degeneration in the monkey pyramidal tract following small discret cortical lesions. *Neu ology* (Minneap.) *20* 89—100 (1970)

DUNC N D A. A relation between axon diameter and myelination determined by measurement of myelinated spinal root fibres. *J Comp Neu ol. 60* 437—471 (1934)

EKHOLM, J Postnatal changes in cutaneous reflexes and in th discharge pattern of cutaneous and articular sense organs. *Act Physiol. Scand* Suppl. 297 1—130 (1967)

FERRARO A. and BARRERA, S. E. Posterior column fibres and their termination in Macacus Rheus. *J Comp Neurol. 62* 507—530 (1935)

FINE, K J B. The nat re and tability of nerve myelin. *I t Rev Cytol. 12* 303—334 (1961)

FLEISCHHAUER, K. and WA TENBERG H. Elektronenmikroskopische U tersuchungen über das Wachstum der Nervenfasern und über das Auftreten von Markscheiden im Corpus callosum der Katze *Z Zellforsch 83* 568—581 (1967)

FORRESTER, J and PETERS, A. Nerve fibres i optic nerv f rat. *N ture* (Lond.) *214* 245— 47 (1967)

FOX, M. W., INMAN, O R. and HIMWICH W A. The postnatal development of the spinal cord of the dog. *J Comp Neurol. 130* 233—240 (1967)

GARDNER, E., LATIMER, F and STILLWELL, D Central connections for afferent fibres from the knee joint of the cat. *Amer J Physiol. 168* 437—441 (1949)

GAZE, R. M. and PETERS, A. The development, structure and composition of the optic nerv of Xenopus laevis. *Quart J Exp Physiol 46* 299—309 (1961)

GLEES, P LIVINGSTON R. B. and SOLER, J Der intraspinale Verlauf nd die Endigungen der sensiblen Wurzeln in den Nucleus gracilis und Nucleus cuneatus. *A ch. Psychiat Nervenkr 187* 190—204 (1951)

GLEES, P and SOLER, J Fibre content of th posterior columns and synaptic connections of nucleus gracilis. *Z Zellforsch. 36* 381—400 (1951)

GRANT G Spinal course and somatotopically localized termination of the spinocerebellar tracts. A experimental study in the cat. *Act Physiol. Scand 193* 1—45 (1962)

GRANT G. and REXED B Dorsal spinal oot afferents t Clarke column. *Brain, 81* 567—576 (1958)

GUTNER, I I Über die Entwicklung der peripheren markhaltigen Nervenfasern. *Z Zellfors h. 25* 259—282 (1936)

GYLLENSTEN L. and MALMFORS, T Myelinization of the optic nerve and its dependence on visual function—a quantitative in estigation in mice. *J Embryol. E p Morph 11* 255—266 (1963)

HA, H. and LIU C. H. Cell origin of the ventral spinocerebellar tract. *J Comp Neurol. 133* 185—206 (1968)

HÄGGQVIST G Analyse der Faserverteilung in einem Rückenmarkquerschnitt (Th3) *Z. Mikrosk.-Anat. Forsch* 39 1—34 (1936)
HÄGGQVIST G Faseranalytische Studien über die Pyramidenbahn. *Acta Psychiat. Neurol.* 12 457—466 (1937)
HESS, A. Postnatal development and maturation of the nerve fibres of the central nervous system. *J Comp Neurol.* 100 461—480 (1954)
HILLEBRAND, H. Quantitative Untersuchungen über postnatale Veränderungen der Glia im Corpus callosum der Katze. *Z. Zellforsch.* 73 303—312 (1966)
HUMPHREY T The development of the pyramidal tracts in human fetuses correlated with cortical differentiation. In *Structure and Function of the Cerebral Cortex* (D B. Tower and J P Schadé, eds.) Elsevier Amsterdam pp. 94—103 (1960)
HURSH, J B. Conduction velocity and diameter of nerve fibres. *Amer J Physiol.* 127 131—139 (1939)
HUTTENLOCHER, P R. Myelination and the development of function in immature pyramidal tract. *Exp Neurol.* 29 405—415 (1970)
JABBUR, S J and BANNA, N R. Presynaptic inhibition of cuneate transmission by widespread cutaneous inputs. *Brain Res.* 10 273—76 (1968)
KARLSSON, U Comparison of the myelin period of peripheral and central origin by electron microscopy *J Ultrastruct Res.* 15 451—468 (1966)
KINGSBURY B. F The "law" of cephalocaudal differential growth in its application to the nervous system. *J Comp. Neurol.* 56 431—464 (1932)
KINGSLEY J R., COLLINS, G. H. and CONVERSE, W K. Effect of sciatic neurectomy on myelinogenesis in the rat spinal cord. *Exp Neurol.* 26 498—508 (1970)
KITAI, S. T and WEINBERG J Tactile discrimination study of the dorsal column-medial lemniscal system and spino-cervico-thalamic tract in cat. *Exp Brain Res.* 6 234—246 (1968)
KRUGER, L. SIMINOFF R. and WITKOWSKY P Single neuron analysis of dorsal column nuclei and spinal nucleus of trigeminal in cat. *J Neurophysiol.* 24 333—349 (1961)
LANGWORTHY O R. A correlated study of the development of reflex activity in fetal and young kittens and the myelinization of tracts in the nervous system. *Carnegie Inst Contrib Embryol.* 20 127—172 (1929)
LAPORTE, Y and LUNDBERG, A. Functional organization of the dorsal spinocerebellar tract in the cat. III. *Acta Physiol. Scand.* 36 204—218 (1956)
LAPORTE, Y LUNDBERG A. and OSCARSSON O Functional organization of the dorsal spinocerebellar tract in the cat. I *Acta Physiol. Scand.* 36 175—187 (1956 a)
LAPORTE, Y LUNDBERG A. and OSCARSSON, O Functional organization of the dorsal spinocerebellar tract in the cat. II. *Acta Physiol. Scand.* 36 188—203 (1956 b)
LASSEK, A. M The human pyramidal tract. IV A study of the mature myelinated fibres of the pyramid. *J Comp. Neurol.* 76 217—225 (1942 a)
LASSEK A M The human pyramidal tract. V Postnatal changes in the axons of the pyramid. *Arch Neurol. Psychiat* 47 422—427 (1942 b)
LASSEK, A M and RASMUSSEN, G L. A comparative fibre and numerical analysis of the pyramidal tract. *J Comp Neurol.* 72 417—428 (1940)
LEHMANN H. J Die Nervenfaser In *Handbuch der mikroskopischen Anatomie des Menschen* (W Möllendorff, ed.) Springer Berlin pp. 515—701 (1959)
LENNOX, M. A Single fibre responses to electrical stimulation in cat's optic tract. *J Neurophysiol.* 21 6—69 (1958)
LLOYD D P C and MCINTYRE, A K. Dorsal column conduction of group I muscle

afferent impulses and their relay through Clarke column. *J Neurophysiol.* *13* 39—54 (1950)

LODIN Z., MARES, V. TREKA, J and STIBRAL, K. Studies on the effect of the histological treatment on nervous tissue III. *Acta Histochem.* (Jena) *33* 295—307 (1969)

LUFT, S. A. *Fixation and embedding of mammalian brain and spinal cord for electron microscopy* *J Ultrastruc. Res.* *4* 108—112 (1960)

MARTY R. and SCHERRER, J Critères de maturation des systemes afférents corticaux. In *Progress in Brain Research* 4 (D P Purpura and J P Schadé, eds.) Elsevier Amsterdam pp. 222—236 (1964)

MATTHEWS, M A. An electron microscopic study of the relationship between axon diameter and the initiation of myelin production in the peripheral nervous system *Anat. Rec.* *161* 337—352 (1968)

MAUNSBACH A. B MADDEN S. C. and LATTA, H Variations in fine structure of renal tubular epithelium under different conditions of fixation. *J Ultrastruct. Res.* *6* 511—530 (1962)

MAXWELL, D S KRUGER, L. and PINEDA, A. The trigeminal nerve root with special reference to the central-peripheral transition zone An electron microscope study in the macaque *Anat. Rec.* *164* 113—126 (1969)

MELLSTRÖM A. and SKOGLUND S Quantitative morphological changes in some spinal cord segments during postnatal development. A study in the cat. *Acta Physiol. Scand.* Suppl. 331 1—84 (1969)

MILART Z. The nervous fibre system of the dorsal funiculi in sheep. *Folia Morph.* (Warsz.) *25* 259—266 (1966)

MORRISON A. The postnatal development of the inferior dental nerve in the cat. *J Anat* *85* 24—35 (1951)

NYBERG-HANSEN R. and RINVIK E. Some comments on the pyramidal tract, with special reference to its individual variations in man. *Acta Neurol. Scand.* *39* 1—30 (1963)

NYSTRÖM B. *Fibre diameter increase in nerves to "slow-red" and "fast white" cat muscles during postnatal development.* *Acta Neurol. Scand.* *44* 265—294 (1968)

NYSTRÖM B. and SKOGLUND S. Calibre spectra of spinal nerves and roots in newborn man. *Acta Morph. Neerl.-Scand.* *6* 115—127 (1965)

OSCARSSON O Functional organization of the ventral spinocerebellar tract in the cat. II. *Acta Physiol. Scand.* *42* Suppl. 146, 1—107 (1957)

OSCARSSON O and UDDENBERG, N Identification of a spinocerebellar tract activated from forelimb afferents in the cat. *Acta Physiol. Scand.* *62* 125—136 (1964)

PALAY S. L., MCGEE-RUSSELL, S M. GORDON, S and GRILLO, M. A. Fixation of neural tissue for electron microscopy by perfusion with solutions of osmium tetroxide *J Cell Biol.* *12* 385—410 (1962)

PEELE, T L *The Neuroanatomic Basis for Clinical Neurology* McGraw Hill, New York, 2nd ed. (1961)

PETERS A. Observations on the connexions between myelin sheaths and glial cells in the optic nerves of young rats *J Anat* *98* 125—134 (1964)

PETIT D and BURGESS, P R. Dorsal column projection of receptors in cat hairy skin supplied by myelinated fibres *J Neurophysiol* *31* 849—855 (1968)

PURPURA, D P SHOFER, R. J HOUSEPIAN, E. M and NOBACK, C. R. Comparative ontogenesis of structure-function relations in cerebral and cerebellar cortex. I *Progress in Brain Research* 4 (D P Purpura and J P Schadé eds.) Elsevier Amsterdam pp 187—221 (1964)

Rexed, B. Contributions to the knowledge of the postnatal development of the peripheral nervous system in man. A study of the bases and scope of systematic investigations into the fibre size in peripheral nerves. *Acta Psychiat. Neurol.* Suppl. 33 1—206 (1944)

Rexed B. A cytoarchitectonic atlas of the spinal cord in the cat. *J Comp. Neurol. 100* 297—380 (1954)

Rexed, B. and Ström, G. Afferent nervous connections of the lateral cervical nucleus. *Act Physiol. Scand. 25* 219—229 (1952)

Roback, H. N and Scherer, H. J Über die feinere Morphologie des frühkindlichen Gehirns unter besonderen Berücksichtigung der Gliaentwicklung. *Virchows A h Path. Anat Physiol. 294* 365—413 (1935)

Romero, C. and Skoglund S. Methodological studies of the technique in measuring nerve fibre diameters. *Acta Morph. Neerl.-Scand 6* 107—114, (1965)

Rushton, W A. H. A theory of the ffects of fibre size in medullated nerve. *J Physiol.* (Lond.) *115* 101—122 (1951)

Ryter, A. and Kellenberger, E. L'inclusion au polyester pour l'ultramicrotomie. *J Ultrastruct. Res.* 200—214 (1958)

Samorajski, T and Friede, R. L. A quantitative electron microscopic study of myelination in the pyramidal tract of rat. *J Comp Neurol. 134* 323—338 (1968)

Schultz R. L. and Karlsson U Fixation of the central nervous system for electron microscopy by aldehyd perfusion. II Effect of osmolarity pH of perfusate and fixative concentration. *J Ultrastruct. Res 12* 187—206 (1965)

Schwieler, G H Respiratory regulation during postnatal development in cats and rabbits and some of ts morphological substrate. *Act Physiol. Scand* Suppl. 304 1—113 (1968)

Sherrington, C S and Laslett E. E. Remarks on the dorsal spinocerebellar tract. *J Physiol.* (Lond.) *24* 188—194 (1903)

Sjöstrand F S. The tructure and formation of the myelin sheath. I *M hanisms of Demyeli ation* 1 (A. S. Rose and C. M. Pearson, eds.) McGraw Hill, New York, pp. 1—43 (1963)

Skoglund S On the postnatal development of postural mechanisms as revealed by electromyography and myography in decerebrate kittens. *Act Physiol. Scand 49* 299—317 (1960)

Skoglund S The spinal transmission of proprioceptive reflexes and the postnatal development of conduction velocity in different hindlimb nerves in th kitten. *Acta Physiol. Scand 49* 318—329 (1960 b)

Skoglund, S. Muscle afferents and motor control in the kitten. I *Muscular Afferents and Motor Control. Nobel Symp I* (R. Granit, ed.) Almqvist & Wiksell, Stockholm pp. 245—259 (1966)

Skoglund, S and Romero, C. Postnatal growth of spinal nerves and roots. *Act Physiol. S and 66* Suppl. 260 1—50 (1965)

Smith D E Observations on the postnatal development of Clarke column in the kitten. *J Comp Neurol 135* 263—274 (1969)

Szentágothai-Schimert J Die Bedeutung des Faserkalibers und der Markscheidendicke im Zentralnervensystem. *Z. Anat. Entwicklung gesch. 111* 201—223 (1941/4)

Terzuolo C R and Terzian H. Cerebellar increase of postural tonus after deafferentiation and labyrinthectomy *J Neur physiol. 16* 551—561 (1953)

TILNEY F and CASAMAJOR, L. Myelinogeny as applied to the study of behaviour. Arch. Neurol. Psychiat. 12 1—66 (1924)
TORACK, R. M. DUFFY M. L. and HAYNES, J. H. The effect of anisotonic media upon cellular ultrastructure in fresh and fixed rat brain. Z. Zellf. sch. 66 690—700 (1966)
UNDERBERG, N. Differential localization in dorsal funiculus of fibres originating from different receptors. Exp. Brain Res. 4 367—376 (1968)
WALKER, A. E. and WEAVER, T. A. The topical organization and termination of the fibres of the posterior columns in Macaca Mulatta. J. Comp. Neurol. 76 145—158 (1942)
WALL, P. D. Two transmission systems for skin sensation. In Sensory Communication (W. A. Rosenblith ed.) Wiley New York pp. 475—496 (1961)
WHITSEL, B. L., PETRUCELLI, L. M. and SAPIRO, G. Modality representation in the lumbar and cervical fasciculus gracilis of squirrel monkeys. Brain Res. 15 67—78 (1969)
WILLIAMS, P. L. and WENDELL-SMITH, C. P. The use of fixed and stained sections in quantitative studies of peripheral nerve. Quart. J. Micr. Sci. 101 43—54 (1960)
WINDLE, W. F. FISH M. W. and O'DONNELL, J. E. Myelogeny of the cat as related to development of fibre tracts and prenatal behaviour patterns. J. Comp. Neurol. 59 139—165 (1934)
WOLFHEKEL, U. Die Übergang der zentralen in die periphere Struktur von Hirnnerven bei den niederen Wirbeltieren. Act. Anat. 74 183—196 (1969)
YOSS, R. E. Studies of the spinal cord. Part II. Topographic localization within the ventral spinocerebellar tract in the macaque. J. Comp. Neurol. 99 613—633 (1953)
ZADUNAISKY, J. A., WALD F. and DE ROBERTIS E. D. P. Osmotic behaviour and ultrastructural modifications in isolated frog brain. Exp. Neurol. 8 290—309 (1963)

Ultrastructural and light-microscopic studies of the nodal region in large myelinated fibres of the adult feline spinal cord white matter

By

Claes Hildebrand

Introduction

The role of the nodes of Ranvier in impulse propagation along myelinated nerve fibres is generally accepted (Tasaki 1939 Huxley and Stämpfli 1949 Hodgkin 1951 Frankenhaeuser 1952 see also Hess and Young 1952) In the largest fibres of adult mammalian peripheral nerves which have the highest conduction velocity (Hursh 1939) the nodal regions display a specialized morphology At the nodes the Schwann cell cytoplasm is differentiated to a collar of regularly arranged microvilli-like processes which pass through an extracellular node gap substance to the immediate vicinity of the nodal axon membrane. Paranodally the myelin sheath is highly crenated and cords of mitochondrion-loaded Schwann cell cytoplasm extend along this region. Each myelinated fibre is enclosed by a continuous basement membrane and is capable of transmitting impulses even when separated from the main nerve trunk by teasing (cf Tasaki 1939 Lehmann 1959) Some major characteristics of the nodal region in large adult mammalian peripheral fibres were revealed by light microscopic studies (see Hess and Young 1952 Lehmann 1959) The nodal-paranodal fine structure in large adult mammalian peripheral fibres has been elucidated by Landon and Williams (1963) Williams and Landon (1963) Berthold and Skoglund (1965) and Berthold (1968 a) Nodes of small peripheral fibres show a much less specialized pattern (Elfvin 1961 Berthold 1968 a)

The existence of nodes of Ranvier in the central nervous system has been repeatedly questioned long after the first light microscopic description by Tourneux and Le Goff in 1875 (for references see Bodian 1951 Hess and Young 1952, Pease 1955 Suhrmann 1962 Bunge 1968) The proposal that nodes are lacking in the central nervous system has even been used as an argument against the theory of saltatory conduction (see Vizoso and Young 1948, Huxley and Stämpfli 1949 Hodgkin 1951) However a wealth of light microscopic reports demonstrate and describe typical nodes in central

nervous tissue. This literature has been reviewed by Lehmann (1959) Sulzmann (1962) and Bunge (1968) Along myelinated central fibres nodes are regularly spaced and, in analogy with peripheral fibres there is a linear relationship between internodal length and fibre size (Bodian 1951 Hess and Young 1952) According to these authors a longer part of the axon is left uncovered by myelin at central than at peripheral nodes and the nodal axon is constricted. The so called "cementing disc of Cajal" and the "spiny bracelets of Nageotte" have been observed at central as well as peripheral nodes (Hess and Young 1952, Lehmann 1959) In myelin sheaths of larger central fibres Schmidt Lanterman incisures are present (Cajal 1928 Feindel *et al.* 1948 Bodian 1951) The central fibres are not individually ensheathed by connective tissue and they are not functional after teasing (see Lehmann 1959)

The ultrastructural literature on myelinated central fibres has recently been reviewed by Bunge (1968) From his review it is clear that the fine structural concept of the central node of Ranvier is based exclusively on studies of very small fibres (1—2 μ) There is only one ultrastructural description of nodes in large central mammalian fibres (Conradi 1969) This is obviously due to the great difficulties in obtaining a satisfactory preservation of large central fibres in particular those of the spinal cord white matter (Luse 1960 Condie *et al* 1961 Bunge *et al* 1961 Conradi 1969) On the basis of the reports cited above describing a highly specialized nodal paranodal pattern in large peripheral fibres in contrast to the comparatively simple organization at nodes of small peripheral fibres, it seemed likely that nodes of large central fibres in a similar manner might be more complex than those of the small fibres so far studied The report by Conradi (1969) on motoraxons within the spinal cord gray matter gave evidence in favour of this assumption.

The aim of the present study is to demonstrate that the nodal regions in large ($\geqslant 5$ μ) fibres within the feline spinal cord white matter exhibit a characteristic architecture which in several respects differs from the appearance of nodes in small fibres and which has not been earlier described

Material and methods

Immersion fixation studies on mammalian spinal cord showed that the myelin sheaths of larger fibres are very difficult to preserve adequately and usually appear severely split, in contrast to the sheaths of smaller fibres (Luse 1960, Condie *et al.* 1961) This was also noted by Bunge *et al* (1961) and by Conradi (1969) after perfusion fixation. Similarly the fixation of large peripheral myelinated fibres is quite another problem than fixation of small peripheral

fibres (see Berthold 1968 b). Since the intention was to study the ultrastructure of the nodal region in large myelinated fibres of the feline spinal cord white matter and since apparently none of the earlier methods rendered and acceptable ultrastructural preservation of such fibres it was necessary first to find a technique suitable for this purpose.

A total of 45 adult cats were used, ranging in weight from 1.8 to 4.5 kg. The animals were anaesthetized by an intraperitoneal injection of 40 mg/kg BW sodium pentobarbital. Below follows a summary of the different fixation methods tried.

In 10 animals the lumbar spinal cord was perfused at a controlled intermittent pressure as described by Berthold (1968 a). The fixative consisted of 5 % glutaraldehyde in a 500 mOsm Millonig buffer with 2.7 % Rheomacrodex (Pharmacia, Sweden) (Berthold 1968 b). In two of these cases a mixture of 5 % CO_2 and 95 % O_2 was used for artificial respiration instead of pure oxygen. It was thought that the CO_2-induced vasodilatation (Kety 1955, Smith *et al.* 1969) might improve the entrance of fixative into the spinal cord. In four animals 0.5 % Xylocaine (Astra, Sweden) was added to the rinsing solution which preceded the perfusate (Forssmann *et al.* 1967).

In 35 adult cats the cervical spinal cord was fixed by perfusion. The operative procedure was similar to that described by Palay *et al.* (196). The animals were respired with 100 % O_2. The rinsing solution (Ringer or Tyrode with 2.7 % Rheomacrodex) and the fixative was perfused into the ascending aorta at the same temperatures and pressures as given by Berthold (1968 b). Immediately prior to or at the onset of perfusion the descending aorta was clamped at the level of the diaphragm. The composition of the perfusate was varied with respect to sort of fixative, fixative concentration and tonicity of the buffer (see Table I). The tonicities were checked with an osmometer (Knauer, Germany).

After perfusion the seventh lumbar or the first cervical spinal cord segment was removed. Pieces of white matter were postfixed in the perfusate, lacking Rheomacrodex, for 3—4 hours, rinsed in a Millonig or veronal acetate buffer, postosmicated in a solution of 2 % OsO_4 in the same buffer (Palade 1952, Millonig 1961) and rinsed again. After dehydration in acetone the pieces of white matter were embedded in Vestopal W (Ryter and Kellenberger 1958). For light microscopic evaluation of the fixation semithin transverse and longitudinal sections were cut with a LKB 4801 Ultrotome. The sections were stained with toluidine blue (Maunsbach *et al.* 1962). The electron microscope was used for the final evaluation.

After perfusion according to Berthold (1968 b) the lumbar spinal cord white matter usually exhibited patches of a yellow-brown colour intermingled

In one of the longitudinally cut series the nodal and internodal (within 10—50 μ from the node) diameters were measured in 38 fibres of varying size. The myelin sheaths were included in the internodal diameters. In Text fig 1 the nodal diameters have been plotted against fibre size The plots approximately follow a straight line within the size range measured. When the fibre size increases from 4 to 16 μ the nodal axon diameter changes from 2 to 5.5 μ If the length of the nodal axon is approximated to 1 μ (see below) without correction for a possible variation with fibre size and assuming that the nodal axon is cylindrical, this would mean that the nodal axon surface area increases from about 6 to 17 μ^2 (cf Berthold 1968 a) Generally the nodal diameter amounts to around 50 % of the internodal fibre size in the smallest and to about 30 % of the internodal diameter in the largest fibres measured.

The axon was constricted both in the node gap and in the zones of myelin sheath termination, at the internodal end of which the transition to a larger diameter took place Within the constricted axon the axoplasm was more dense than elsewhere and contained small dark granules. This was most pronounced in the largest fibres (Fig 1) In longitudinal sections the node gap appeared as a narrow fairly straight slot best seen in sections passing tangentially to the nodal axon (Figs. 2 3 and 4 b) The distance separating the inturning myelin sheaths was about 1 μ or less. In very small fibres the node gap seemed wider (Fig 6)

The longitudinal dimension of the myelin decrement zones, as seen in the light microscope remained fairly constant at 3—4 μ in spite of varying fibre size and myelin sheath thickness. Outside the decrement zones of the inturning myelin sheaths it was common to find one or a few large compact bodies with a staining similar to myelin. These bodies were roughly spherical and ranged in diameter from 3 to 6 μ

In cross sections through the nodal region the constricted axon had an almost perfect circular outline in contrast to the rather wavy contour internodally In cross sections through the decrement zone the outer contour of the myelin sheath was irregular and the compact myelin like bodies were more outstanding than in longitudinal sections. The position of the compact bodies close to the node often corresponded to outbulgings or irregularities of the myelin sheath contour in sections at a greater distance from the node (Figs. 7 8 and 9) Close to the node the diameter of the whole fibre became reduced and th space left outside the fibre was occupied by glial cytoplasm. In sections passing close to or through the node gap the fibre was separated from surrounding fibres by a mantle of glial cytoplasm (Figs. 7 8 and 9) Sometimes an astrocyte was located in the neighbourhood of the node (Fig 7) No counterpart to the characteristic ridges and grooves with associated cords of

Schwann cell cytoplasm in paranodes of large peripheral fibres could be found not even in the largest central fibres. However as stated above, the outer contour of the myelin sheath often appeared irregular close to the node and in particular at the decrement zones (Figs. 7 8 and 9)

To sum up, the large (≥ 5 μ) central fibres displayed a light microscopic nodal-paranodal morphology which differed from the appearance of nodes in large peripheral fibres in the following respects

1 No counterpart to the paranodal arrangement in large peripheral fibres with cords of Schwann cell cytoplasm and myelin sheath crenation was observed in the present material

2. The characteristic large myelin like bodies which were present at central nodes have not been described in the peripheral nervous system.

3 The perinodal region between the two inturning myelin sheaths outside the node gap is occupied by a mantle of glial cytoplasm at central nodes In peripheral nerve fibres this region contains connective tissue and is delimited from the node gap by a basement membrane.

Electron microscopy

General description

In the electron microscope the wide range of fibre sizes, the predominance of small myelinated fibres and the relatively sparse glial cytoplasm in the investigated area of white matter was striking Frequently myelin sheaths of different fibres apposed each other with the formation of an intraperiod line at the contact zone (Fig 11) (cf Peters 1960 196., Walberg 1963) Within the axoplasm of all fibres neurofilaments and neurotubules, with diameters of 50—70 Å and 200—250 Å respectively were present. In the larger fibres the proportion of neurofilaments was higher than in small fibres as in peripheral nerves (Friede and Samorajski 1970) A moderate number of elongated mitochondria and scattered vesicular profiles occurred regularly in the axoplasm. Very few unmyelinated fibres were encountered. For the present study the interest was focused on fibres with a diameter of 5 μ or more

The myelin period was estimated in 50 cross-sectioned fibres of one cat. In order to avoid errors from the compression of the sections caused by sectioning (Karlsson 1966) the measurements were limited to parts of the myelin sheaths where the myelin lamellae were parallel to the cutting direction. The central myelin period as measured between the midpoints of two successive major dense lines amounted to 85—115 Å (cf Maturana 1960 Peters 1962, Karlsson 1966 among others) As also noted by Karlsson (1966) the lamellar spacing

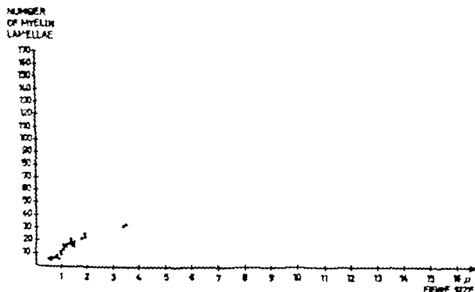
varied within one and the same myelin sheath, the period being slightly greater in curved than in straight parts of the sheath. Furthermore a closer spacing was found in the myelin sheaths of large fibres (85—100 Å) than in the sheaths of very small fibres (100—115 Å). The difference in myelin period was best seen where a small fibre directly apposed a large fibre (Figs. 10 and 11). A very dense material often appeared in the major dense lines (Fig. 20) and small localized widenings of the intraperiod line were also observed. The latter were however never radially arranged as described in the rat and mouse optic nerve (Peters 1962, 1964).

Incisures of Schmidt and Lanterman were common in large fibres (Blakemore 1969, Conradi 1969). They were formed by accumulations of cytoplasm within widenings of the major dense line (Fig. 19). The cytoplasmic portions of the outermost and innermost lamellae were larger and less electrondense than those in between, which appeared very dense (Figs. 19 and 20). In the less dense cytoplasmic portions microtubules (200 Å) could be observed. Similar microtubules were present in the outer and inner cytoplasmic tongue of the myelin sheath. In peripheral fibres the incisures follow a straight and regular course through the whole myelin sheath thickness at an angle of 9° to the longitudinal axis of the fibre (Friede and Samorajski 1969). In thick central myelin sheaths they usually present a zig-zag pattern (Fig. 19) as also noted by Conradi (1969). Furthermore the incisures did not always extend all the way from the outermost to the innermost myelin lamella (Fig. 19).

Astrocytic extensions, characterized by bundles of filaments (70—80 Å) (Wolff 1965) frequently apposed the external surface of the myelin sheath (Peters 1962, Walberg 1965, Lumsden 1968). In the periphery of the astrocytic processes a moderate number of partly dense-cored microtubules (200 Å) (cf. Rodriguez Echandia *et al.* 1968, Raine and Wisniewski 1970) were present. Groups of gliosomes (see Blinzinger *et al.* 1964, Mugnaini and Walberg 1964, Wendell-Smith *et al.* 1966, Hashimoto 1966) were prominent in the astrocytic cell-body and major processes but less common in the pericapillary extensions. Some gliosomes were rounded or ovoid with a longest diameter of 0.5—6 μ and narrow elongated forms were also seen. Very large gliosomes were present in the sub-pial astrocytes. *The gliosomes were bounded by two triple-layered* membranes from the inner of which a number of crista-like structures projected into a dense granular or fibrous matrix (Figs. 15—18). (For a more detailed description of gliosomes see Gray 1959, 1960, Srebro 1965, Hashimoto 1966, 1969, Schonbach 1969). The oligodendrocytes had a structure similar to that described in earlier reports (Mugnaini and Walberg 1964, Kruger and Maxwell 1966, see also Bunge 1968). Direct continuity between oligodendrocytes and mature myelin sheaths (Hirano 1968) could be observed in some cases.

where the cell-body was located close to or surrounded the fibre (Figs. 22 and 23). Glial cells similar to microglia were also encountered either as perivascular cells or within the nervous tissue. They were rather infrequent and nearly always contained polymorphous dense bodies and what appeared as lipid inclusions (cf. Vaughn and Peters 1968, Stensaas and Stensaas 1968, Mori and Leblond 1969). Some microglial cells were associated with large compact bodies with a diameter up to 7 μ (Figs. 13 and 14). These bodies displayed a fairly uniform lamellar substructure with a repeating period around 45—50 Å (Fig. 1[illegible]).

In cross sections of specimens from one cat the number of myelin lamellae in each of 64 fibres of varying sizes was counted in the electron microscope (when less than 40 lamellae) or on the plate (when 40 lamellae or more) at a high magnification. Each fibre was photographed at a low magnification and the diameter (including the myelin sheath) was measured on the plate. The results are illustrated in Text fig. 2. The number of myelin lamellae increases with increasing fibre size in a linear fashion (cf. Samorajski and Friede 1968). As the fibre size changes from 0.5 to 16 μ the myelin lamellae increase in number from 5 to around 165. As seen from the graph a rough estimate of the number of myelin lamellae in the sheaths of the measured fibres can be



Text-fig. 2. Number of myelin lamellae plotted against fibre size (myelin sheath included) in 64 fibres from the lateral funiculus in the first cervical spinal cord segment of one adult cat.

obtained by multiplying the fibre size by 10 Thus the myelin sheath of a fibre with an external diameter of 10 μ will contain about 100 lamellae. Since the central myelin period is approximately 100 Å (85—115) (see above and Karlsson 1966) the sheath thickness should amount to 1 μ From this follows that the axon diameter is around 8 μ The quotient between axon diameter and fibre size will thus be 0.8 (cf Rushton 1951 Stoeckenius and Zeiger 1956, Lehmann 1959)

The decrement zone

On each side of the node the uncoiling of the myelin sheath occupied a length of 3—5 μ in accordance with the light microscopic observations. The terminal cytoplasmic pockets derived from the innermost and outermost myelin lamellae were larger and less electron-dense that the majority of pockets (Fig 24) Within the less dense pockets usually 2—4 microtubules (200 Å) could be seen. The cytoplasm of the small dense pockets contained a highly electron-dense material (Figs. 26 and 28) Some pockets contained rounded membrane-bounded bodies At the internodal end of the decrement zone all terminal cytoplasmic pockets apposed the axon membrane but in the more nodal parts of the decrement zone only a fraction of the pockets contacted the axon The remainder formed double piles which with an internodal tilt extended for a varying distance into the inturning myelin sheath (Figs. 25—28) (cf Conradi 1969) This pattern is similar to that in the corresponding region of large peripheral fibres (Berthold 1968 a) This arrangement of the terminal cytoplasmic pockets most likely corresponds to the "spiny bracelets of Nageotte observed by the earlier light microscopists (see Hess and Young 1952) Usually the pockets derived from the outermost myelin lamellae did not contact the axon but formed a row of pockets overhanging the node gap and thus constituted the lateral walls of this space (Fig 24) The extracellular space of the node gap extended in between the deepest overhanging pocket and the axon as a small wedge-shaped recess (Fig 24) (cf Berthold 1968 a)

The shape of each terminal cytoplasmic pocket can be compared with that of a hanging drop as seen in a longitudinal section (Figs. 26 and 28) Most pockets had a width of 0.04—0.06 μ and a length which varied somewhat but as a rule measured 0.15 μ Each terminal cytoplasmic pocket which apposed the axon covered a length of about 0.10—0.15 μ Commonly the cytoplasm of several consecutive pockets continued as thin sheets into the major dense lines of the corresponding myelin lamellae Such stacks of widened myelin lamellae usually were curved (Fig 25) Where pockets apposed the axon membrane a 7-layered membrane complex was formed (Fig. 27) The periodical thickenings in the outer leaflet of the axon membrane described by

others in the decrement zone of very small central fibres (Andres 1965 Kruger and Maxwell 1966, Laatsch and Cowan 1966, Peters 1966) were never observed.

In the internodal end of the decrement zone axonal evaginations protruded into the pockets and conversely the pockets partly invaginated the axon. This was most pronounced in the largest fibres (Fig 29) It is noteworthy that this complex relationship between the axon and the cytoplasmic components of its myelin sheath is located precisely where the axon diameter is becoming reduced. Throughout the rest of the decrement zone the axon was straight and uniform in diameter

In the light microscope large compact bodies which stained like myelin were often seen at the nodes. Ultrastructurally these bodies were made up of concentric somewhat undulating lamellae forming a thick compact layer around a center of very dense cytoplasm (Fig 3?) Both in the center and at the periphery of the bodies fissures and empty spaces, probably representing shrinkage artefacts, were regular features. A closer examination revealed that as a rule the lamellar pattern differed from that of myelin Major and minor dense lines were not clearly discernible but the dense lines were uniform (Fig 33) Consequently the repeating period was less than that of myelin amounting to 45—50 A. The name myelinoid body" is suggested for this structure which was first observed by Conradi (1969) No direct continuity between the lamellae of the inturning myelin sheath and those of the juxtaposed myelinoid body could be observed. As mentioned above microglial cells sometimes contained or surrounded bodies with a similar appearance in addition to various dense bodies of smaller size and different structure (Figs. 12, 13 and 14)

Th nodal paranodal ax n

As stated above the axon diameter is reduced at the internodal end of the decrement zone and remains constricted throughout the decrement zone In the node gap the nodal axon often bulged out slightly to achieve a roughly biconical shape in longitudinal sections. Here as in the decrement zone, the axon appeared almost perfectly circular in cross sections in contrast to the irregularly rounded shape of the paranodal axon beyond the decrement zone (Figs. 32 and 35) The length of the nodal axon measured 0.8—1 1 μ (cf Conradi 1969) On the axoplasmic side the nodal axon membrane was coated by a 250—350 A thick moderately dense granular layer (Peters 1966 Conradi 1969) which was separated from the inner leaflet of the nodal axon membrane by a narrow (60—100 A) light zone (Fig 3 a) The inner coating of the axon membrane was absent in the decrement zone but present in the lateral recesses of the node gap. A moderate number of spherical invaginations (750—850 A)

were regularly noted in the nodal axon membrane At these sites the dense coating was attenuated or absent (cf Berthold 1968 a)

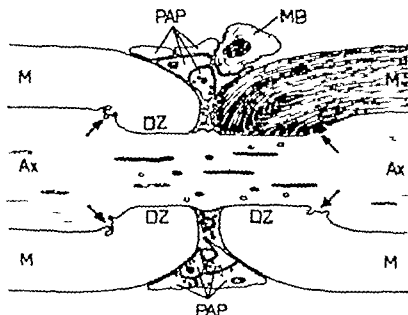
In the constricted axon segment neurotubules and neurofilaments were tightly packed and several mitochondrial profiles as well as numerous empty spherical vesicles were present. The vesicles were of the same size as the aforementioned nodal axon membrane invaginations. In addition a few dense-cored vesicles, some lamellar bodies (0 1—0.2 μ) and small irregular membranous profiles could be observed This contrasts to the comparatively empty appearance of the internodal axon (Figs. 24 and 32)

The node gap and the perinodal region

In the light microscope a mantle of pale blue glial cytoplasm was observed occupying the immediate surroundings of the nodes of large fibres, but the origin of this cytoplasm remained uncertain. Ultrastructurally the perinodal cytoplasm could be identified as fibrous astrocytic processes containing gliosomes, bundles of filaments and some partly dense-cored microtubules. In the following these processes will be denoted as PAP (perinodal astrocytic processes) The description below refers mainly to studies of transverse sections. The PAP were arranged in a plane approximately perpendicular to the longitudinal axis of the fibre and filled the perinodal region between the two inturning myelin sheaths as well as the peripheral parts of the node gap (Figs. 24 32 and 35) The perinodal astrocytic processes which faced the nodal axon were separated from it by a finely granular extracellular matrix. Tufts of long slender microvilli like processes emerged from these PAP and passed through the matrix substance of the node gap to the immediate vicinity of the nodal axon membrane (Figs. 32 and 35) The microvillous processes had a diameter of 500—600 Å and contained a moderately dense cytoplasm in which a few thin filaments sometimes could be seen. Apart from the microvillous tufts the cytoplasmic expansions of the PAP gave rise to tubule-containing foot like processes, the flattened ends of which passed perpendicularly to the longitudinal axis of the axon close to the nodal axon membrane (Fig 32) The microvillous processes and in particular the foot like processes tended to point into the lateral recesses of the node gap and thereby came in close spatial relation to the overhanging terminal cytoplasmic pockets. This feature necessitated a very careful examination of the series in order to exclude a possible oligodendroglial contribution to the cytoplasmic contents of the node gap However according to the present observations, the microvillous tufts and the foot like processes were exclusively of astrocytic origin. In longitudinal sections the astrocytic nodal extensions appeared as discontinuous irregular profiles (Figs. 24 30 and 31) The extracellular nodal matrix extended in a labyrin-

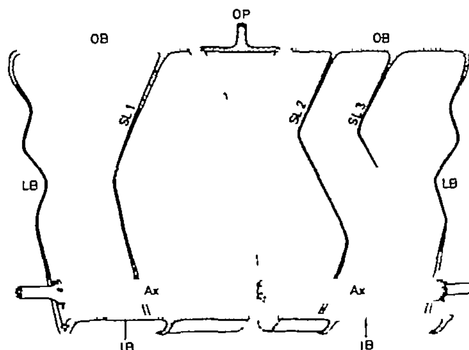
thue fashion between the PAP sometimes several microns away The perinod al astrocytic processes often contained some gliosomes but no correlate to the *paranodal accumulations of mitochondria at nodes of large peripheral fibres* (Williams and Landon 1963 Berthold and Skoglund 1965 1967 Berthold 1968 a) could be found.

As noted in the light microscope an astrocytic cell body sometimes was located in the neighbourhood of a node In these instances the juxtanodal astrocyte contributed to the perinodal astrocytic processes of that node and in a few cases one astrocyte seemed to take part in the formation of PAP at two or three neighbouring nodes of Ranvier (Fig. 33) The present observations do not permit any certain statement as to whether the PAP at one node may be derived from more than one astrocyte although this seems likely



Text-fig. 5 *Diagrammatic representation of the nodal region of large central fibre.* The axon (Ax) is constricted t the nod and in the decrement zones (DZ) At the node the membrane bulges out slightly The nodal axoplasm contains vesicles dense lamellated bodies and some elongated mitochondria. In the upper right decrement zone the arrangement of the terminal cytoplasmic pockets can be seen. At the internodal ends of the decrement zones axonal evaginations are present (arrows) Perinodal fibrous astrocytic processes (PAP) give rise to delicat processes hich pass through an extracellular nod gap substance to the vicinity of the nodal axon membrane. The lateral walls of the node gap are formed by overhanging terminal cytoplasmic pockets. Outsid the paranodal myelin sheath (M) a myeloid body (MB) is present.

In Text fig 3 the principal arrangements at large central nodes of Ranvier are schematically depicted. Text fig 4 represents a hypothetical explanation of some features of the decrement zone and the incisures of Schmidt and Lanterman by a modification of the drawing by Hirano and Dembitzer (1967)



Text-fig 4 Schematic dr wing modified from Hirano and Dembitzer (1967) showing the myelin sheath of one internode in an unrolled state OB = outer and IB = inner cytoplasmic border f the myelin sheath. OP = ligodendroglial process joining the outer cytoplasmic border LB = lateral cytoplasmic border which in th original tat coils around the paranodal axon. The axon shows fingerlike evaginations which protrude into the first turns of the coiling lateral cytoplasmic border More nodally the axon is constricted and the part of the lateral border coiling around the constricted axon is thin and dense An undulant course of the lateral border in this region would correspond t the double piles of small and dense terminal cytoplasmic pockets seen in longitudinal sections through decrement zones of large fibres. The last part of the lateral cytoplasmic border (upper part of picture) forms the overhanging terminal cytoplasmic pockets which face the node gap. The outer and inner cytoplasmic borders are interconnected by thin bands of cytoplasm (SL 1 and SL 2) which in the rolled state form the incisures of Schmidt and Lanterman In longitudinal section SL 1 will appear V-shaped, SL 2 will follow zig zag course through the myelin and SL 3 will be incomplete extending only through the outer layers of the myelin sheath It is stressed that the arrangements in the picture are hypothetical and that the size proportions are highly distorted.

The difference in period between myelin sheaths of very small and large fibres, might have some connection with the variations in chemical composition of myelin from different places within the central nervous system (Wolfgram and Kotorii 1968, Mehl and Wolfgram 1969) assuming that myelin from a region exclusively composed of small fibres has another chemical composition than myelin from a region also including large fibres (Amaducci *et al.* 1962).

However the varying period could also be an artefact caused by a different influence of the fixative and dehydrating agents upon thick and thin myelin sheaths. Another possibility is that the higher degree of myelin sheath curvature in small fibres as compared to large fibres might influence the myelin period (cf. Karlsson 1966). The significance and nature of the myelinoid bodies is as yet not understood. A relevant discussion with regard to them will have to await further studies.

The most important of the findings reported here is that the nodal morphology of large central fibres is much more complex than that of small central fibres described by others (see Bunge 1968).

The constriction of the axon in the nodal region of large central fibres already observed by the early light microscopists (see Hess and Young 1952) was more marked the larger the fibre. This, in connection with the constant longitudinal dimension of the decrement zone, means that the area of contact between the constricted axon and the terminating myelin sheath does not increase in proportion to the fibre size (cf. Berthold 1968 a). Obviously the limited longitudinal dimension of the decrement zone will not allow apposition of more than 25—40 terminal cytoplasmic pockets to the axon, since each pocket occupies a length of 0.10—0.15 μ. Thus when the number of myelin lamellae exceeds 25—40, which is the case at a fibre size over 4 μ, all pockets cannot appose the axon, which could account for the double piles of terminal cytoplasmic pockets in decrement zones of large fibres.

Interdigitations between the axon and the terminal cytoplasmic pockets at the internodal end of the decrement zone have also been described in large peripheral fibres (Berthold 1968 a). This arrangement seems to represent a localized increase of the contact area between the axon and the cytoplasmic compartments of the myelin sheath located precisely where the axon diameter is becoming reduced.

The length of the nodal axon amounted to around 1 μ, as also found by Conradi (1969) in central motoraxons and Berthold (1968 a) in large peripheral fibres. The constant longitudinal dimension of the nodal axon and the increasing degree of constriction with increasing fibre size should keep the

area of the nodal axon membrane exposed at the node within certain limits (cf Berthold 1968 a) Since the nodal axon membrane is allotted an important role in the theory of saltatory conduction (Tasaki 1939 Hodgkin 1951) the dimensions of the nodal axon should have functional significance. The specific design and limited dimensions of the two regions bordering the node, the decrement zones, indicate that they might fulfill a specific function. The possibility that the decrement zones are involved in the physiological events during impulse propagation has been discussed by Hess and Young (1952) in connection with peripheral nerve fibres.

In the constricted part of the nodal-paranodal axon neurotubules and neurofilaments were densely packed and numerous other organelles were found in this region in contrast to the rather empty appearance of the internodal axon. The phenomenon of axoplasmic transport, (Taylor and Weiss 1965 Weiss and Holland 1967 Lasek 1968 a, b Mc Ewen and Grafstein 1968 Karlsson and Sjöstrand 1968 Sjöstrand and Karlsson 1969 Chou 1970) raises the question how the transported material passes the narrow crowded nodal axon. As proposed by Weiss *et al* (1964) and Grafstein *et al* (1970) the neurotubules represent a possible transport route.

The presence of an extracellular matrix in the mammalian central nervous system has been demonstrated with the aid of different techniques (Hess 1953 1955 Uzman and Rumley 1958 Young and Abood 1960 Clausen and Hansen 1963 Bondareff 1966 Pease 1966 Rambourg and Leblond 1967 Palladini and Alfei 1968) Ultrastructurally the central fibres exhibit a node gap substance which is most abundant at the nodes of large fibres (Andres 1965 Peters 1966, Karlsson 1967 Conradi 1969) Two different mucopolysaccharides which resemble chondroitin sulphate and hyaluronic acid have been found in central nervous tissue (see Saigo and Egami 1970)

Astrocytic extensions have been observed at nodes of large mammalian central fibres by Conradi (1969) However the architecture described in the present report with filament-loaded perinodal astrocytic processes from which tufts of microvillous processes and foot-like cytoplasmic extensions passed through the node gap substance to about on the nodal axon membrane has not been reported earlier Astrocytes in tissue culture secrete a PAS-positive material from the tips of their processes (Lumsden 1968 cf Dorfman and Ho 1970) into the extracellular space and glycogen—a possible precursor—is a well known constituent of this cell (see Mugnaini and Walberg 1964 Lumsden 1968) Thus one function of the nodal astrocytic extensions might be to produce and maintain the node gap substance.

At peripheral nodes a node gap substance is likewise present in which the Schwann cell microvilli are embedded and according to Langley and Landon

(1967) this matrix contains a sulphated mucopolysaccharide In a recent report the peripheral nodal matrix was characterized as a "sponge of anionic centres capable of mopping up cations in the vicinity and exchanging them for others" (Langley 1969) Experimental evidence was presented to support the idea that K expelled from the axon could displace Na from this "ionic sink" It seems likely that the perinodal matrix at central nodes in a similar way may act as a cationic exchange resin There is also physiological and histochemical evidence that the astrocyte is involved in the regulation of the water-ion balance of the extracellular space in the central nervous system (see Friede 1965 and review by Kuffler and Nicholls 1966) In conclusion it is suggested that the astrocyte produces the nodal matrix and may influence the milieu of the node gap by specific processes at this site. The increasing nodal complexity with increasing fibre size might reflect higher functional demands on larger fibres.

In the peripheral nervous system the nodal microvillous collar to which the astrocytic microvillous tufts demonstrated here obviously are a counterpart is *continuous with the paranodal mitochondrion-loaded Schwann cell cytoplasm* (Landon and Williams 1963, Williams and Landon 1963 Berthold 1968 a) The possibility that the paranodal mitochondria are involved in the postulated function of regulating the nodal milieu attributed to the nodal microvillous collar in peripheral fibres has been discussed (Landon and Williams 1963 Williams and Landon 1963 Wendell-Smith *et al* 1965 Berthold and Skoglund 1967 Berthold 1968 a) However no juxtanodal accumulations of mitochondria were found at central nodes (cf Friede *et al* 1963 Conradi 1969) This could be explained by the different general organization of the white matter In a peripheral fibre one Schwann cell extends over the whole internodium which may be 1.5 mm in length (Vizoso and Young 1948) and the mitochondria are concentrated to the *circumnuclear and paranodal areas* The distance between a central node and the nearest astrocyte can hardly amount to millimeters. On the contrary astrocytic cell bodies were sometimes observed in the vicinity of nodes. The supposed energy demands could be supplied from the astrocytic cell-body and its major processes where groups of gliosomes are found. One astrocyte might serve a number of nodes within the range of its processes although such an arrangement was not proved

Gliosomes are generally believed to represent a variety of mitochondria (Gray 1960 Wendell-Smith *et al.* 1966, Srebro 1965 Mugnaini and Walberg 1964 Schonbach 1969 Hashimoto 1966, 1969) They exhibit a somewhat unusual structure, but crista like formations and double limiting membranes indicate their mitochondrial nature. Maybe the difference in structure between gliosomes and conventional mitochondria reflects a difference in metabolic

function According to Friede (1965) the supply of oxidative enzymes is small in normal astrocytes whereas oligodendrocytes show a higher activity On the other hand astrocytes, in contrast to oligodendrocytes, respond to changes in the environmental ion concentration by an extreme increase of oxidative enzymes (Friede 1965)

In addition to possible nodal functions the Schwann cell also maintains a myelin sheath In the central nervous system the role of the Schwann cell seems to be played by at least two cells. The astrocyte as discussed above is possibly concerned in regulating the nodal extracellular milieu (cf Kuffler and Nicholls 1966) and producing the matrix of the node gap (cf Lumsden 1968) while the oligodendrocyte maintains one or a number of myelin sheaths (see Bunge 1968 Peters and Proskauer 1970) That the latter function is energy-consuming is indicated by the high metabolic activity (see Elliott and Heller 1957) and the relatively numerous mitochondria in oligodendrocytes (Mugnaini and Walberg 1964)

Finally the discovery of a complex nodal-paranodal structure in large adult central fibres as opposed to the comparatively simple morphology of nodes in small adult central fibres (see Bunge 1968) evokes the question at which fibre size the nodes of developing future large, central fibres have attained mature structural characteristics.

Summary

The nodal region of large ($\geqslant 5\ \mu$) myelinated fibres in the feline spinal cord white matter was studied by light and electron microscopy The animals were fixed by glutaraldehyde perfusion. The number of myelin lamellae was found to be linearly related to fibre size by a factor of 10 In myelin sheaths of very small fibres the period was greater than in large fibres. The incisures of Schmidt and Lanterman were usually irregular forming a zig zag pattern and they did not always extend through the entire myelin sheath thickness. No counterpart to the paranodal myelin sheath crenation and accumulations of Schwann cell mitochondria in peripheral nerves was found in central fibres. Outside the paranodal myelin sheath large compact lamellated bodies with a lamellar pattern different from that of myelin commonly occurred. Similar bodies were sometimes contained within microglia. At the nodes the axon was constricted to 30—50 % of the internodal fibre size and the axoplasm was more dense than elsewhere. In the internodal end of the decrement zones axonal evaginations protruded into the terminal cytoplasmic pockets. The decrement zones occupied a length of around 4 μ of the axon on each side of a node. Only

a restricted number of terminal cytoplasmic pockets apposed the axon the remainder being piled up onto each other The length of the nodal axon was about 1 μ Perinodal astrocytic processes gave rise to microvilli-like and foot like processes which projected through the extracellular node gap substance to the vicinity of the nodal axon membrane. The findings were compared with the results from studies of nodes in large peripheral fibres and some functional implications were discussed

Acknowledgements

This investigation was supported by grants from Karolinska Institutet (Reservationsanslaget) and from the Swedish Medical Research Council (projects no B70-14X 136-06 and B71 14X 136-07A) I am much indebted to Professor Sten Skoglund for his encouragement and constructive criticism and to Mrs. Ingegerd Lindqvist and Miss M J Berghman for excellent technical assistance

References

AMADUCCI, L. PAZZAGLI, A. and PESSINA, G The relation of proteolipids and phosphatidopeptides to tissue elements in the bovine nervous system. *J N ochem.* 9 309—318 (1962)

ANDRES, K. H. Über die Feinstruktur besonderer Einrichtungen in markhaltigen Nervenfasern des Kleinhirns der Ratte *Z Zellforsch.* 65 701—712 (1965)

BERTHOLD C. H. Ultrastructure of the nodo-paranode region of mature feline ventral lumbar spinal-root fibres. *Act Soc M d. Upsal* 73 37—78 (1968)

BERTHOLD C. H. A study on the fixation of large mature feline myelinated ventral lumbar spinal-root fibres. *A ta. Soc M d Upsal.* 9 1—56 (1968 b)

BERTHOLD C. H. and SKOGLUND S Ultrastructure and histochemistry of the developing node of Ranvier in the hindlimb nerves of the cat. *A ta. Soc M d Upsal.* 70 287—293 (1965)

BERTHOLD C. H and SKOGLUND S. Histochemical and ultrastructural demonstration of mitochondria in the paranodal region of developing feline spinal roots and nerves *A t Soc M d Upsal* 7 37—70 (1967)

BLAKEMORE, W F Schmidt Lanterman incisures in the central nervous system. *J Ultrastruct Res* 29 496—498 (1969)

BLUNSCHORN, K., RAWCASTLE, N B and HAGER, H Untersuchungen zur genaueren Analyse des Feinbaus einer in Astrocyten des Goldhamstergehirns vorkommenden besonderen Mitochondrienart (Prisma Typ) *Proc 3rd Eur p R g. Conf on El. mic ool B* 283—284 (1964)

BODIAN D A note on nodes of Ranvier in the central nervous system. *J Comp Neurol* 94 475—484 (1951)

BONDAREFF W An intercellular substance in rat cerebral cortex Submicroscopic distribution of ruthenium red *Anat Rec* 157 527—536 (1967)

Brody I The keratinization of epidermal cells of normal guinea pig skin as revealed by electron microscopy *J Ultrastruct Res.* 2 482—511 (1959)
Bunge, R. P Glial cells and the central myelin sheath *Physiol. Rev* 48 197—251 (1968)
Bunge, M B Bunge R. P and Ris, H Ultrastructural study of remyelination in an experimental lesion in adult cat spinal cord. *J Biophys. Biochem. Cytol.* 10 67—94 (1961)
Chou S M Axoplasmic flow rates in the monkey optic sy tem. *Neurology* (Minneap.) 96 607—612 (1970)
Clausen J and Hansen A. Acid mucopolysaccharides of human brain Identification by means of infra red analysis. *J Neurochem.* 10 165—168 (1963)
Conder, R. M., Howell, A. E. and Good R. A Studies on the problem of preservation of myelin sheath ultrastructure evaluation of fixation, dehydration and embedding techniques. *J Biophys. Biochem Cytol.* 9 429—443 (1961)
Conradi S. Observations on the ultrastruct re of the axon hillock and initial axon segment of lumbosacral motoneurons i the cat. *Acta Physiol. Scand* Suppl. 332, 65—84 (1969)
Dorfman, A. and Ho P L. Sy thesis of acid mucopolysaccharides by glial tumour cells in tissue culture *Proc Nat Acad Sci. U.S* 66 495—499 (1970)
Elfvin L.-G The ultra tructure of the nodes of Ranvier in cat sympathetic nerve fibres *J Ult astru t R s* 5 374—387 (1961)
Elliott K. A. C. and Heller, I H. Metabolism of neurons and glia. I *M tabolism I the Ne vous System* (D Richter ed.) Pergamon Press, London, pp. 286—290 (1957)
Feindel, W H Allison, A. C. and Weddell, G Intravenous methylene blue for ex perimental studies on the central nervous system. *J Neurol. Neu su g Psychiat* 11 227—239 (1948)
Forssmann W G Siegrist G Orci L., Girardier, L., Pictet R. and Rouiller, C. Fixation pa perfusion pour la microscopie electronique *J Microsc p* 6 279—304 (1967)
Frankenhaeuser, B. The hypothesis of saltatory conduction. *Cold Spring Harbor Symp. Q ant Biol.* 17 27—36 (1952)
Friede R. L. Enzyme histochemistry of neuroglia. In *Prog ess in Brain Resea h* 15 (E. D P De Robertis and R. Carrea eds.) Elsevier Amsterdam pp 35—47 (1965)
Friede, R. L. Fleming, L. and Knoller, M A comparati e study of enzymes involved in hexose monophosphate shunt and citric acid cy le in the brain. *J Neurochem.* 10 263—277 (1963)
Friede, R L and Samorajski T The clefts of Schmidt Lanterman. A quantitative electron microscopic study of their struct re in developing and adult sciatic nerve of the rat. *Anat Rec* 165 89—102 (1969)
Friede, R L and Samorajski T Axon caliber related to neurofilaments and micro-tubules in sciatic nerve fibres of rats and mice *Anat Rec* 167 379—388 (1970)
Galey F R and Nilsson S. E. A new method for transferring sections from the liquid surface of the trough through staining solutions to the supporting film of the grid. *J Ultrastru t Res* 14 405—410 (1966)
Grafstein B McEwen B S. and Shelanski M L. Axonal transport of neurotubule protein. *Nature* 227 289—290 (1970)

Gray E. G. Electron microscopy of neuroglial fibrils of the cerebral cortex. *J Biophys. Biochem Cytol. 6* 1 1—122 (1959)
Gray E. O Reticular organization of material in certain mitochondria of neuroglia of lizard brain. *J Biophys Biochem. Cytol 8* 282—285 (1960)
Hamimoto, P H. Gliosome and its relation to astrocytic filaments in cat brain. In *6 th Int Congr for Micr* A) *t 2* (R. Uyeda ed.) Maruzen Comp Tokyo, pp. 467—468 (1966)
Hamimoto P H Electron microscopic studies of gliosome formation in postnatal development of the spinal cord in the cat. *J Comp Neurol. 137* 51—255 (1969)
Hess, A. The ground substance of the central nervous system revealed by histochemical staining. *J Comp Neu ol 98* 69—9? (1953)
Hess, A. Blood-brain barrier and ground substance of central nervous system. Similarities in development. *Arch Neu ol Psychiat 73* 380—386 (1955)
Hess A. and Young J Z. The nodes of Ranvier *Proc Roy Soc B 140* 301—320 (195)
Hildebrand C. and Skoglund S. Calibre spectra of some fibre tracts in the feline central nervous system during postnatal development. *A ta Phy iol S and* Suppl. 364 5—42 (1971)
Hirano A. A confirmation of the oligodendroglial origin of myelin in the adult rat. *J Cell Biol. 38* 637—640 (1968)
Hirano, A. and Dembitzer, H M. A truct ral analy is of the myelin sheath in the central nervous system. *J Cell Biol. 34* 555—567 (1967)
Hirano A. and Dembitzer, H. M. The transverse bands as means of acces t the periaxonal space of the central myelinated nerve fiber *J Ult astruct Re 28* 141—149 (1969)
Hodgkin A. L. The ionic basis of electrical activity in nerve and muscle. *Biol. Reviews* 26 339—409 (1951)
Hursh, J B. Conduction velocity and diameter of nerve fibres *Amer J Physiol 127* 131—139 (1939)
Huxley A. F and St mpfli, R. E idence of saltatory conduction in peripheral my linated nerve fibres. *J Physiol* (London) *108* 315—339 (1949)
Karlsson J.-O and Sjöstrand J Transport of labelled proteins in the optic nerve and tract of the rabbit. *Brain R 11* 431—439 (1968)
Karlsson U L. Comparison of the myelin period of peripheral and central origin by electron microscopy *J Ult ast t R 15* 451—468 (1966)
Karlsson, U L. Three-dimensional tudies of neurons in the lateral geniculate ucleus of the rat. III Specialized neuronal contacts in the neuropile *J Ultrastruct Res 17* 137—157 (1967)
Kety S S Blood flow and metabolism of the human brain in health and disease. In *Neurochemistry* (K. A. C. Elliott, I H. Page and J H. Quastel eds.) Thomas Springfield pp 294—310 (1955)
Kruger, L. and Maxwell, D Electron microscopy of oligodendrocytes in normal rat cerebrum. *Amer J A at 118* 411—437 (1966)
Kuffler, S. W and Nicholls J G The physiology of neuroglia. *Ergebn. Physiol. 57* 1—90 (1966)
Laatsch, R. H. and Cowan W M A structural pecialization at nodes of Ranvier in the central nervous system *Nat 210* 757—758 (1966)

5—715870. Hildebrand

LANDON D N and WILLIAMS P L. Ultrastructure of the node of Ranvier \ tu 199 575—577 (1963)

LANGLEY O K. Ion exchange at the node of Ranvier *Histochem J 1* 295—309 (1969)

LANGLEY O K. and LANDON D N A light and lectron histochemical approach t the node of Ranvier and myelin of peripheral nerv fibres. *J Histochem Cytochem 15* 722—731 (1967)

L sEK, R. J Axoplasmic transport of labelled protein in rat ventral motor neurons. *E p \ u ol 21* 41—51 (1968 a)

LA K, R. J \xoplasmic transport in cat dorsal root ganglion cells as studied with ³H L-leucine. *Brain Res. 7* 360—377 (1968 b)

LEHMA N H J Die Nervenfaser In *Ha db ch d m k osk pisch A tom der Mensche* (W v Möllendorff ed.) Springer Berlin pp. 515—701 (1959)

LUMSDEN C. E. Nervous tissue in cult re In *The Stru t d F nction of Nervous Tiss I St uct* (G H Bourne ed) Academic Press New York, pp 67—140 (1968)

LUSE, S. A. Fixation and embedding of mammalian brain and spinal cord for electron microscopy *J Ultrastruct Res 4* 108—112 (1960)

MATURANA, H R. The fine anatomy of the optic nerve of anuran \n electron microscope study *J Bi phys Biochem Cytol. 7* 107—120 (1960)

MAUNSBACH A. B. MADDEX S C. and LATTA, H Variations in fine structure of renal tubular epithelium under different conditions of fixation. *J Ult astruct R s. 6* 511—550 (1962)

McEWEN B S. and GRAFSTEIN B. Fast and low components in axonal transport of protein. *J C ll Biol. 38* 494—508 (1968)

MEHL, E. and WOLFGRAM F Myelin types with different protein components in the same species. *J Neurochem. 16* 1091—1097 (1969)

MILLONIG G Advantages of a phosphat buffer for OsO sol tions in f xation. *J Appl. Phys 32* 1637 (1961)

MORI, S. and LEBLOND C. P Identification of microglia in light and electron microscopy *J Comp Neu ol 135* 57—80 (1969)

M GNAINI E. and WALBERG F Ultrastructure of neuroglia. *Ergebn Anat E tw l l gsgesch 37* 194—236 (1964)

PALADE, G E. \ study of fixation for lectron microscopy *J Exp Med 95* 285—297 (1952)

PALLADINI, G and ALFEI, L. Histochimie d neuropile d poulet pendant l'ontogenèse — hyperstrié vral. *Histochemie 14* 314—323 (1968)

PALAY S L. McGEE-RUSSELL, S M. GORDON S. and GRILLO, M. A Fixation of neural tissue for lectron microscopy by perfusion w th sol tions of osmium tetroxide. *J C ll Biol 12* 385—410 (1962)

PEASE, D C. Nodes of Ranvier in the central nervous system. *J Comp Neu ol. 103* 11—15 (1955)

PEASE, D C Polysaccharides associated with the exterior surf ce of epithelial cells kidney intestine brain. *J Ult astruct Res 15* 555—588 (1966)

PETERS, A. The formation and str ct re of myelin sheaths in the central nervous system. *J Biophys Biochem Cytol. 8* 431—446 (1960)

PETERS, A Plasma membrane contacts in the central nervous system. *J A t 96* 237—48 (196)

PETERS A. Further observations on the structure of my lin sheaths in the entral nervous system. *J Cell Biol.* 20 281—296 (1964)

PETERS, A. The node of Ranvier in the central nervous system. *Quart J E p Physiol* 51 229—236 (1966)

PETERS, A. and PROSKAUER C. C. The ratio between myelin segments and oligodendrocytes in the optic nerve of the adult rat *Anat Rec* 163 243 A (1969)

RAINE, C. S. and WISNIEWSKI, H. On the occurrence of microtubules w thin matur astrocytes *Anat R* 167 303—308 (1970)

RAMBOURG, A. and LEBLOND C. P Electron microscopic observ tions on the carbohydrate-rich cell coat present at the surface of cells in the rat. *J Cell Biol.* 32 27—53 (1967)

REYNOLDS, E. The use of lead itrate at high pH a an electron opaque tain in lectron microscopy *J Cell Biol.* 17 208—212 (1963)

RODRIGUEZ ECHANDIA, E. L., PIEZZI, R. S. and RODRIGUEZ E. M. Dense-core microtubules in neurons and gliocytes of the toad. *Amer J Anat.* 122 157—168 (1968)

RUSHTON W A. H A theory of the effects of fibre size in medullated nerve. *J Phy iol* (London) 115 101—122 (1951)

RYTER, A. and KELLENBERGER, E L inclusion u polyester pou l' ltramicrotomie *J Ult astru t Res.* 2 200—214 (1958)

SAIGO K. and EG MI, F Purification and some properties of acid mucopolysac harides of bovine brain. *J Neu ochem* 17 633—647 (1970)

SAMORAJSKI, T and FRIEDE, R. L. A quantitati lectron microscopic study of my lination in the pyramidal tract of rat *J Comp N urol* 134 323—338 (1968)

SCHONBACH C. The neuroglia in the spinal cord of the newt, Triturus irideseens. *J Comp Neurol* 135 93—120 (1969)

SJÖSTRAND J and KARLSSON J-O Axoplasmic transport in the optic nerve and tract of the rabbit A biochemical and radioautographic tudy *J Neurochem* 16 833—844 (1969)

SMITH, A., PEARCE, J W nd ALEXANDER, S. C. Effects of P_{CO_2} on spinal cord blood flow *Amer J Physiol* 16 1158—1163 (1969)

SKERLO Z. The ultrastructur of gliosomes in the brains of amphibia. *J C ll Biol* 261 313—322 (1965)

STENSAAS, L J and STENSAAS S S Astrocytic neuroglial cells, oligodendrocytes and microgliacytes in the spinal cord of the toad. II Electron microscopy *Z Zellfors h* 86 184— 13 (1968)

STOECKENIUS W and ZEIGER, K. Morphologie der segmentierten Nervenfaser *E geb Anat Entwicklungsgesch* 35 420— 531 (1956)

SULLEMANN R Die mikroskopische Morphologie der zentralen markhaltigen Nervenfaser *Wiss. Z Friedri h-S hiller U Jen Math. Nat Reih* 11 197— 45 (1962)

TASAKI, I. The electro-saltatory transmission of the nerve impulse and the ffect of narcosis pon the nerv fiber *Amer J Physiol.* 127 211—227 (1939)

TAYLOR, A. C nd WEISS P Demonstration of axonal flow by the movement of tritium-labelled protein in mature optic nerv fibres. *Proc N t Acad S U.S* 54 15 1—15.7 (1965)

TOURNEUX, F and LE GOF R. Note su les étranglements des tubes nerveux de la moelle épinière. *J Anat Physiol (Paris)* 11 403—404 (1875)

UZMAN L. L. and RUMLEY M K. Changes in the composition of the developing mouse brain during early myelination *J Neu ochem* 3 170—184 (1958)

VAUGHN J E. and PETERS, A. A third neuroglial cell type An electron microscopic study *J Comp Neurol 133* 269—288 (1968)

VIZOZO A. D and YOUNG, J Z. Internod length and fibre diameter i developing and regenerating nerves. *J A at* (London) 82 110—134 (1948)

WALBERG F An electron microscopic study of the inferior olive in the cat *J Comp. Neurol. 120* 1—18 (1963)

WEISS P and HOLLAND Y Neuronal dynamics nd axonal flow II The olfactory nerve as model test object. *Proc Nat Acad Sci. U.S 57* 258—264 (1967)

WEISS, P TAYLOR, A. C. and PILLAI P A The nerv fibre as a system in continuous flow: Microcinematographic and lectron microscopic demonstrations. *Scie e 136* 330 (1962)

WENDELL-SMITH C. P BLUNT M J and BALDWIN F The ultrastructural characterization of macroglial cell types. *J Comp N urol. 127* 219—240 (1966)

WENDELL SMITH C. P BLUNT M J BALDWIN F and PAISLEY P B Neurone-satellite cell relationship. *Nature 205* 781—782 (1965)

WILLIAMS, P L. and LANDON D N Paranodal apparatus of peripheral myelinated nerve fibres of mammals. *N t 198* 670—673 (1963)

WOLFF J Elektronenmikroskopische Untersuchungen über Struktur und Gestalt von Astrocytenfortsätzen. *Z Z llforsch 66* 811—828 (1965)

WOLFGRAM F and KOTORII, K. The composition of the myelin proteins of the central nervous system. *J Neu ochem 15* 1281—1290 (1968)

YOUNG, I J and ABOOD, L. G Histological demonstration of hyaluronic acid in the central nervous system. *J Neurochem 6* 89—91 (1960)

Legends to figures

Figs. 1—9 are light micrographs and Figs. 10—35 electron micrographs.

Fig 1 Longitudinal section. Nodal-paranodal region of large fibre Th axon (Ax) is constricted at the node and in the decrement zones. Note the density of the nodal axoplasm. The terminating myelin sheaths turn in toward the axon and between them the narrow and deep node gap (arrows) can be seen. (2000 x)

Figs 2 and 3 Longitudinal tangential sections through the nodal-paranodal regions of tw large fibres. The node gap appears as a narrow slot between the terminating myelin sheaths (arrows) (2000 x)

Fig. 4 a and b Longitudinal sections from the nodal-paranodal region of the same fibr Note the axonal constriction nd the axoplasmic dens ty in Fig. 4 a and the narrow node gap (arrows) in the tangential section (Fig. 4 b) (2000 x)

Figs. 5 and 6. Longitudinal sections from the nodal-paranodal region in mediumsized and small fibre respectively Arrows point to the node gap. Compare Fig 1 and Fig. 4 a. (2000 x)

Fig. 7 a—e. Series of transverse sections through the nodal-paranodal egion of large fibre (Axon indicated by x) The first section (Fig. 7) is lose t the node gap passing through the decrement zone The axon is circular and contains some granules. A large myelin-like body (1) is attached outside the myelin sheath. An astrocytic nucleus is seen in the right top corner (N) and the fibre is surrounded by glial cytoplasm Fig. 7 b One section later Part of the nod gap is in luded (arrow) Fig 7 c. Section number four in the series. The plane of section passes through the decrement zone on the other ide of the node gap. A new my lin-like body has appeared (2) Fig 7 d. Section number nine The axon diameter has increased and the axoplasm is less dense Fig. 7 e Section number 19 approximately 9 u f om the node gap Observe the change in size and shape of the fibre The astrocytic nucleus is t ll present (N) (2000 x)

Fig. 8. Transverse section th ough the nodal-paranodal region of smaller fibre Note the circular shape of the axon (x) the irregular outer ontou of the myelin sheath and the presence of glial cytoplasm around the fibre (2000 x)

Fig 9 a—c. Series of transverse sections through the nodal-paranodal region of large fibre. (Axon indicated by x) Fig. 9 a. Section umber one close to the node gap Note the myelin-like bodies (1 2 and 3) nd the perinodal glial cytoplasm. Fig 9 b. Section number three through the decrement zone Fig 9 Fifteen sections later Note the change in size and shape of the fibre The myelin heath outbulgings (arrow) are located at the place of the dense myelin-like bodies in earlier sections. The glial cytoplasm has been reduced and is largely limited to the icinity of the capillary (cap) in the upper part of the pict re (2000 x)

Figs. 10 nd 11 Details of my lin sheaths of medium-sized and large (right) and very small (left) fibres. Note the difference in period. Arrows point to inner cytoplasmic tongue of the medium-sized fibre in Fig 10 and outer cytoplasmic tongue of the ery small fibre in Fig. 11 (115 250 x and 124 600 x espectively)

Fig. 12 Detail of Fig 13 showing lamellar pattern of microglial inclusion. Compare Figs. 10 and 11 The lamellar pa tern is different f om that in myelin. (124 600 x)

Fig. 13 Longitudinal section. Microglial cell close t my linated fibre (nucleus indicated by N) Same cell as in Fig 20 Note the large inclusion (arrow) The inclusion is lamellated and the lamellar pattern can be seen in Fig 12 which is a high magnification of the area indicated by the rectangle Numerous lipid droplets are also present (L) (11,500 ×)

Fig 14 Longitudinal section. Group of microglial cells (nuclei indicated by N) surround a large body (×) with a lamellar pattern as in Fig 1 (5000 ×)

Figs. 15 16 17 and 18 Transverse sections showing arious forms of gliosomes. Note the cristae in Figs. 15 and 18 (arrows) and the ordered internal gliosome structure in Figs. 16 and 17 (46 700 51,500 4_,700 and 51,500 × respectively)

Fig. 19 Longitudinal section through Schmidt Lanterman incisure The incisure follows a zig-zag pathw y Note the density of the incisural cytoplasmic portions and their continuity with the major dense lines in the myelin (small arrows) Large arrow indicates where the incisure is interrupted. (115,_50 ×)

Fig 20 Longitudinal section. Straight simple Schmidt Lanterman incisure in medium-sized fibre (Ax = axon) I the my lin dense areas can be seen (arrows) Inset show detail of such area. A highly electron dense material is located to the major dense lines. Note the microglial cell (Mg) (12,600 ×) Inset (115,_50 ×)

Fig. 1 Longitudinal section. Detail of Schmidt-Lanterman incisure One of the cytoplasmic portions contains rounded body (×) An lectronopaque material is present between some of the other cytoplasmic portions (arrows) This location corresponds to the minor dense lines of the myelin. (115,_50 ×)

Fig 22. Cross section. An oligodendroglial process (OP) is spiraling around a myelinated fibre (arrows) (1_,200 ×)

Fig 23 Cross section. An oligodendrocyte (nucleus indicated by N) is apposed to a my linated fibre This cell has a process which spirals around the fibre (long arrow) and gradually becomes transformed into myelin (short arrow) (23,000 ×)

Fig 4 Longitudinal section. Node of Ranvier The nodal axon (AX) contains dense axoplasm w th several vesicles, some small dense bodies, which at a higher magnification were lamellated, and few elongated mitochondria (M) The terminal cytoplasmic pockets appear ery dense except in the nodal and internodal ends of the decrement zones Note the pockets which overhang the node gap. Perinodal astrocytic processes (PAP) giv rise to d licat extensions (short arrow) which pass into the node gap (15 150 ×)

Fig. 5 Longitudinal section. Node of Ranvier (AX = axon, NG = node gap) Small arrows point to terminal cytoplasmic pockets in the decrement zones. Note how some pockets ppose the axon whereas other pile up ont ach other Large arrow indicate tacks of curved myelin lamellae the major dense lines of which ar widened containing a dense cytoplasm continuous with that of terminal cytoplasmic pockets. (31,050 ×)

Fig. 26 Longitudinal section. Detail of decrement zone showing the general arrangement of terminal cytoplasmic pockets (AX = axon) (86,800 ×)

Fig. 7 Longitudinal section. Detail of decrement zone (AX = axon) The seven-layered membrane omplex where terminal cytoplasmic pockets ppose the axon can be seen at some places (arrows) (115,_50 ×)

Fig. 28. Longitudinal section. Detail of decrement zone showing pile of terminal cytoplasmic pockets. It is clearly seen how successive myelin lamellae end as pocket (indicated by figures 1—9) the cytoplasm of which is continuous with the major dense lines of the myelin lamellae (arrow) (113,250 ×)

Fig. 29 Longitudinal section. The picture shows axonal evaginations into the internodal end of a decrement zone (small arrows) (AX = axon) Large arrow points in nodal direction (12,000 ×)

Fig. 30 Longitudinal tangential section through node gap (NG) (MY = myelin sheaths) Several microvilli-like processes (small arrow) and an irregular larger cytoplasmic process (P) occupy the node gap. (4 ,000 ×)

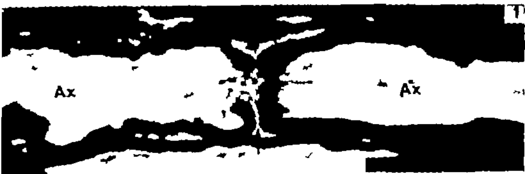
Fig. 31 Longitudinal section. Node of Ranvier (AX = axon) An astrocytic process (AP) sends out a tapering extension (arrow) which points into the node gap (NG) (21,500 ×)

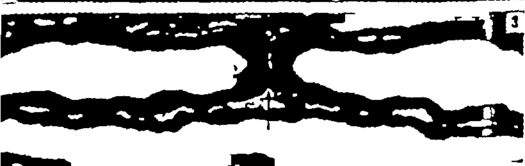
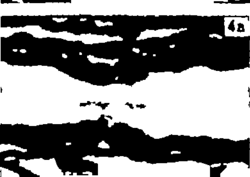
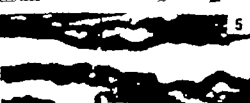
Fig. 32 a, b and c Series of transverse sections through node of Ranvier Fig. 32 a. The nodal axon (AX) contains many vesicles and mitochondria (m) in addition to various other bodies Note the close packing of neurofilaments and neurotubules. The nodal axon membrane has a dense appearance Inset shows higher magnification of nodal axon membrane (AX = axon) Note the dense material on the axoplasmic side of the membrane (arrows) In the large picture the axon is apposed by terminating myelin (MY) on the left side. A finely granular material surrounds the uncovered nodal axon and separates the nodal axon membrane from perinodal astrocytic processes (PAP) The latter contain filaments and some gliosomes (G) The perinodal astrocytic processes give rise to tufts of microvilli-like processes (small arrows) and tubule-containing foot-like processes (FP) In the upper right corner a myelinoid body can be seen (MB) Note the dense compact outer layer and the more dense but less compact interior (17 400 ×) Fig 32 b and c. Later in the series. In Fig. 32 c the terminating myelin on the other side of the node is beginning to appear (MY) Note the astrocytic foot like process (FP) which in Fig. 32 points in between the axon and the terminating myelin. This location corresponds to the lateral recess of the node gap (13,800 ×)

Fig. 33 Detail of myelinoid body showing the outer compact layer Note the difference between the lamellar pattern of the myelinoid body and that of normal myelin (inset) (113,250 ×)

Fig 34 Detail of myelinoid body showing the internal structure. A dense membrane bounded cytoplasm can be seen (×) (63 400 ×)

Fig. 35. Cross section through node of Ranvier (AX = nodal axon) Part of the axon circumference is apposed by terminating myelin (MY) Outside the uncovered part of the axon finely granular extracellular material can be seen Perinodal astrocytic processes (PAP) give rise to microvilli-like processes (arrows) which pass through the nodal extracellular material to the immediate vicinity of the nodal axon membrane. One of the perinodal astrocytic processes originates from the astrocyte in the upper part of the picture (nucleus indicated by N) Note the large gliosomes (G) Processes of the same cell also appear to be present at another node in the lower left corner of the picture (×) (15 150 ×)


1
Ax
Ax

2

3

4a

5

4b

6


7a
X
1


7b
X
1


7c
2
X
1


7d
N
X


7e
N
X


8

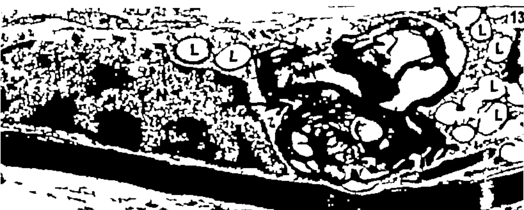

cap
9a
1
X
2
3


cap
9b
1
X
2
3


cap
9c
X


10

11

12

13
L
L
L
L
L
L

14

15

16

17

18




AX








25
AX


26
AX


27
AX

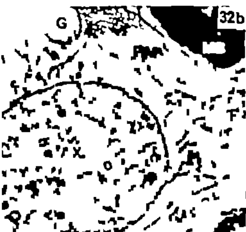

28
1
2


29


30
MY
MY
P
P

31
AP
NG
AX

32a
G
MY
MB
AX
FP
G
PAF
AK



32b
G
MB



32c
G
MB
MY
AX



33



34
X



35
G
G
G
MY
AX
AP

pockets and the abundant nodal extracellular matrix in adult motoraxon nodes (Conradi 1969) On the other hand the length of the node gap and the longitudinal dimension of the decrement zones was the same as in the adult (Conradi and Skoglund 1969) These findings partly resembled the pattern in nodes of developing peripheral nerves (Berthold 1968 a)

In a previous report describing the nodal region of large fibres in the adult feline spinal cord white matter (Hildebrand 1971) the presence of a highly specialized architecture was demonstrated. Astrocytic extensions encircled the constricted nodal axon and tufts of microvilli-like and foot like processes passed from the perinodal astrocytic processes through a node gap substance to the immediate vicinity of the nodal axon membrane. At the decrement zones double piles of terminal cytoplasmic pockets were found and large compact myelinoid bodies were present outside the inturning myelin sheaths.

Recent studies have demonstrated that the nodal-paranodal Schwann cell specializations in large adult peripheral fibres (Williams and Landon 1963 Landon and Williams 1963 Berthold and Skoglund 1965 Berthold 1968 b) are not established in developing fibres until they have attained a diameter around 4 μ (Berthold and Skoglund 1967 1968 a, b Berthold 1968 a) At this size the fibres are close to functionally mature (see Discussion in Skoglund and Romero 1965 and Berthold 1968 a) Moreover evidence has been presented that a rapid increase in conduction velocity of developing central fibres is initiated at a fibre size around 4 μ (Purpura *et al.* 1964)

Against this background is seemed worthwhile to examine the postnatal structural development of the nodes of Ranvier in future large fibres in the white matter and in particular to find out at which fibre size an essentially adult pattern is established. It was assumed that the initiation of a rapid increase in conduction velocity of developing central fibres at a diameter around 4 μ mentioned above might be correlated to a structural maturation of the active sites in impulse propagation—the nodes of Ranvier (Tasaki 1939 Huxley and Stampfli 1949 Hodgkin 1951)

Material and methods

15 kittens in ages varying from one day to 3 months were used. The kittens were anaesthetized by an intraperitoneal injection of 20—40 mg/kg sodium pentobarbital and the cervical spinal cord was fixed by vascular perfusion with 5 % glutaraldehyde in a 300 mOsm Millonig buffer (pH 7.2—7.4) For details on the perfusion procedure which is a modification of the method

described by Berthold (1968 c) for fixation of the spinal roots, see Hildebrand (1971). The first cervical spinal cord segment was removed and cut into 1 mm thick transverse slices. Each slice was divided by a median cut and trimmed to include only the lateral funiculus. After postfixation for 3—4 hours in the perfusate lacking Rheomacrodex, the specimens were rinsed for 1—2 hours in a 300 mOsm veronal-acetate or Millonig buffer, immersed for 3—4 hours in 2 % osmium tetroxide in the veronal acetate or Millonig buffer (Palade 1952, Millonig 1961) followed by 30 minutes rinse in the buffer. The solutions were cold and the pH was 7.2—7.4. After dehydration in acetone the specimens were embedded in Vestopal W (Ryter and Kellenberger 1958). Transverse and longitudinal semithin (0.5 μ) sections from the superficial part of the lateral funiculus were cut in series on a LKB 4801 Ultrotome and stained with toluidine blue (Maunsbach *et al.* 1962) for light microscopic examination. For electron microscopy series of 100—200 consecutive transverse and longitudinal thin sections with a grayish interference colour were cut from the same region. The thin sections were placed in sequential order on one hole copper grids (Galey and Nilsson 1966) coated with carbon stabilized formvar. After staining with uranyl acetate (Brody 1959) and lead citrate (Reynolds 1963) the series of sections were examined in a Philips EM 300 electron microscope.

Results

The investigation was limited to the larger fibres in the superficial region of the lateral funiculus in the first cervical spinal cord segment. This corresponds to the location of the spinocerebellar pathways (Grant 1962, Oscarsson and Uddenberg 1964). Since the same area was used for a study of adult central nodes of Ranvier (Hildebrand 1971) and for measurements of fibre sizes during the postnatal development (Hildebrand and Skoglund 1971) comparisons are possible. Those myelinated fibres which at early developmental stages were the largest are considered to be the future large fibres (cf. Hess 1954, Samorajski and Friede 1968).

Light microscopy

In newborn kittens, where the largest fibres of the investigated area with few exceptions measure around 2 μ (Hildebrand and Skoglund 1971), the nodes of Ranvier were recognized with some difficulty. Generally the myelin sheaths

were irregular and the fibres followed an undulant course (Fig 1) At the nodes the terminating myelin sheaths did not turn in towards the axon but were straight or turned out from the axon At both ends of the uncovered nodal axon the row of terminal cytoplasmic pockets appeared as a diffusely outlined collar which was thicker than the myelin sheath. The nodal axon was practically invisible being of the same low density as the internodal axon. The axon appeared slightly reduced in diameter at the node (Figs. 1 and 2) Irregular or rounded fragments resembling myelin were common outside the decrement zones and the myelin sheaths often showed paranodal protrusions.

At the end of the second week the nodes of the largest fibres (around 3 μ) were more distinctly demarcated. In addition to a more pronounced constriction of the nodal axon an increased nodal axoplasmic density was discernible (Fig 3) Fig 7 shows cross-sections through the nodal region. The series of sections clearly shows the presence of large compact myelin-like bodies outside the paranodal myelin sheath.

In three week old kittens the size of the largest fibres was 4—5 μ (Hildebrand and Skoglund 1971) Here the terminating myelin sheaths turned slightly inward at both sides of the node and the decrement zone rendered a more compact impression. The constriction and the increased axoplasmic density of the nodal-paranodal axon was clearly visible (Fig 4)

In 4—6 week old kittens the nodes of fibres measuring 5—6 μ or more presented a basically mature pattern (see Hildebrand 1971) Here the fibres followed a fairly straight course but as in the three week old animals irregularities in the myelin sheath and myelin-like bodies were common close to the nodes. At the nodes the myelin sheaths turned in towards the axon and the cytoplasmic compartments of the myelin sheath at the decrement zone were not distinguishable from the rest of the myelin sheath. The nodal paranodal axon was straight and clearly constricted with a fairly dense axoplasm (Fig. 5) The node gap was more narrow and deep than at earlier stages and appeared as a narrow slot in tangential sections.

By the age of three months the largest fibres measure over 10 μ (Hildebrand and Skoglund 1971) and the nodal regions displayed the same general appearance as in the adult (Fig 6) (Hildebrand 1971)

Thus the light microscopic observations show that the nodes of future large fibres in the investigated area of spinal cord white matter appear highly immature at birth corresponding to a fibre size around 2 μ. During the third postnatal week the mature nodal configuration is beginning to appear in 3—5 μ fibres and at later stages the nodes of Ranvier present a basically mature picture in fibres which have attained a size of 5—6 μ or more

First week In the newborn stage most fibres were unmyelinated At the nodes of Ranvier of the large (about 2 μ) fibres the axon was slightly constricted and straight in the decrement zones but bulged out in the node gap (Fig 8) In some of the largest fibres the axoplasm was slightly more dense at the node as compared to the internode. A few mitochondria, occasional lamellar dense bodies and a few spherical empty vesicles could be seen in the nodal axon At the level of the decrement zone the axon appeared smooth and circular in cross sections (Fig 11) but was irregular in the node gap (Fig 12) As in the adult (Hildebrand 1971) the nodal axon membrane was coated on the axoplasmic side by a moderately dense granular material (Fig 12 Inset)

At both ends of the node gap the decrement zones occupied a length of 3 0 to 4.5 The terminal cytoplasmic pockets were fairly large and of a low electron density as compared to the mature stage (Fig 10) No measurements were attempted on the single pockets since their size, shape and length of apposition varied (Fig 8) Within the pocket cytoplasm some microtubules, a few dense rod-shaped mitochondria and occasional dense bodies could be observed. All terminal cytoplasmic pockets apposed the axon and the successive pockets formed a row along the axon. Only incidentally one or a few pockets were separated from the axon by their neighbours. As in the adult a 7-layered membrane complex was formed where pockets apposed the axon. The periodical thickenings in the outer leaflet of the axon membrane described by others (Laatsch and Cowan 1966, Peters 1966) in nodes of small adul central fibres were never observed. Where successive pockets contacted each other desmosome like formations commonly occurred close to the axon (Fig 10)

The number of pockets in the two adjoining decrement zones at a node was almost never the same One of the decrement zones was usually composed of 2—10 pockets more than the other This was a large difference since at this stage of development the decrement zone comprised at the most about 20 pockets. Consequently the myelin sheath at one side of a node could be thicker than on the other side.

Frequently the row of pockets was continued in a nodal direction by one to seven cytoplasmic portions with an internal structure similar to that of the pockets but lacking continuity with myelin lamellae as seen from longitudinal sections. The apposition between a cytoplasmic portion and the axon was identical with the pocket axon junction and the dense undercoating of the nodal axon membrane was absent underneath the cytoplasmic portions (Figs. 8 9 and 13) From tangential longitudinal sections it was seen that these formations represented a finger like cytoplasmic process which coiled around

the axon (Fig. 13) The process appeared to be an extension from the last turn of the uncoiling cytoplasmic spiral which formed the nodal end of the myelin sheath. This arrangement increased the length of the decrement zone and reduced the length of the uncovered nodal axon (Fig. 13)

As stated above the terminal cytoplasmic pockets varied in size and shape. In some instances the most nodally situated pockets were very large and bulging almost obliterating the node gap. In such pockets a lamellated and granular material was commonly seen (Fig 14) Commonly compact lamellar bodies similar to myelinoid bodies (Hildebrand 1971) were associated with the decrement zones of the larger fibres (Fig 15) In some of the largest fibres axonal evaginations protruded into the most internodal pockets. In a few instances the axon was distorted by multiple evaginations in the decrement zone (Fig 16) Furthermore nodes with an abnormal appearance could be seen. In some cases several consecutive pockets in the nodal part of the decrement zone appeared to be peeled off from the axon by extensions from the more internodally situated pockets (Fig 17) In other instances the two decrement zones facing each other at a node were partly overlapping with the result that the node was closed (Fig 18) (cf Bunge *et al.* 1961) Nodes where the uncovered nodal axon was several microns long were also encountered. Sometimes the nodal axon was filled with lamellated bodies (Fig 19)

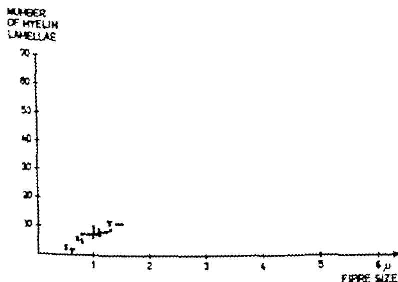
In spite of varying number of terminal cytoplasmic pockets and cytoplasmic portions the longitudinal dimension of the uncovered nodal axon was fairly constant measuring 0.5—1.3 μ (most commonly 0.8—0.9 μ) The abnormal nodes mentioned above represent an interesting exception. The nodal axon was surrounded by a comparatively large extracellular space containing small aggregates of a flocculent material (Figs. 8 and 12) In some of the largest fibres astrocytic extensions were present within the perinodal region and the node gap but the characteristic arrangement of microvillous tufts and foot like processes present at nodes of large mature central fibres (Hildebrand 1971) was not established.

To sum up the nodal ultrastructure of μ fibres in the most immature animals in several respects reminds one of the description of nodes in similarly sized adult central fibres given by others (see Bunge 1968) In contrast to the nodes in small adult fibres the node gap as a rule measured close to 1 μ and a cytoplasmic process often continued the spiral formed by the uncoiling myelin sheath. Generally the nodal structure of future large fibres was very simple in the most immature animals as compared to the appearance of nodes in large adult fibres (Hildebrand 1971)

Second week During this period the myelinated fibres increased in size and sheath thickness and at the end of the second week the largest fibres measured around 3 μ (Hildebrand and Skoglund 1971) Concomitantly with the growth in myelin sheath thickness the number of terminal cytoplasmic pockets increased, but the longitudinal dimension of the decrement zones remained at approximately 4 μ Similarly the length of the uncovered nodal axon remained close to 1 μ At this stage the nodal axon was clearly constricted in many fibres. The axoplasm was denser nodally than internodally (Fig 20) although less pronounced than in the adult Axonal evaginations into the most internodal part of the decrement zone were noted in some fibres.

In some fibres the nodal axon was surrounded by astrocytic extensions from which slender processes extended into the node gap (Fig 20) In the nodal region as well as generally in the white matter the extracellular space seemed to be reduced during the second week. The amount of nodal extracellular matrix was still very scanty Outside the decrement zones whorls of myelin and compact lamellar bodies resembling myelinoid bodies (Hildebrand 1971) frequently occurred

In conclusion the nodal ultrastructure changed slightly towards maturity with the growth in fibre size. The length of the nodal axon and of the decrement zone remained largely unchanged.



Text-fig. 1 Number of myelin lamellae plotted against fibre size (myelin sheath included) in 75 fibres from the lateral funiculus in the first cervical spinal cord segment of one three week old kitten.

Third week During this period the diameter of the largest fibres increased to 4—5 μ (Hildebrand and Skoglund 1971) and these fibres became comparatively heavily myelinated.

In cross-sections of specimens from one three week old kitten the number of myelin lamellae was counted and the fibre size (myelin sheath included) was measured in 73 fibres which ranged in size from 0.5 to 5.25 μ The measurements and countings were performed as described in a previous study (Hildebrand 1971) The results are illustrated in Text fig 1 The number of myelin lamellae increased with fibre size in a linear fashion (Samorajski and Friede 1968) from 5 lamellae or less at around 0.5 μ to 50—60 lamellae at 4—5 μ No myelinated fibres with a diameter less than 0.5 μ were observed (cf Fleischhauer and Wartenberg 1967) In an earlier paper on adult central fibres (Hildebrand 1971) it was stated that a rough estimate of the number of myelin lamellae could be obtained by multiplying the fibre size in microns by 10 In the three week old kitten the number of myelin lamellae increased slightly more rapidly with fibre size than in the adult (cf Friede and Samorajski 1967 Samorajski and Friede 1968) and the smallest myelinated fibres of the kitten (0.5 μ) had fewer lamellae than similarly sized fibres of the adult cat. The material is however much too limited to permit any definite statements on this point.

In the decrement zones of large fibres the terminal cytoplasmic pockets appeared more dense and had smaller dimensions than at earlier stages. As in the adult the most nodally and internodally located pockets were larger and less dense than the pockets in between. A few overhanging pockets could be seen in the node gap and the cytoplasmic portions which at earlier stages continued the row of pockets, were now absent. Some pockets did not appose the axon membrane but formed small sequestered groups (Figs. 21 and 24) Both the longitudinal dimension of the decrement zone and the length of the uncovered nodal axon remained largely unchanged at around 4 μ and 1 μ respectively At the nodes the axon diameter was approximately half the internodal fibre size (myelin sheath included)

In cross sections the nodal axon had an approximately circular outline although localized outgrowths occurred (Fig 23) The nodal axon contained several vesicles, closely spaced neurotubules and neurofilaments, some mitochondria and a few lamellated dense bodies. As a consequence of the increased myelin sheath thickness the node gap was deeper than at earlier stages. Perinodally many astrocytic processes were present containing filaments, microtubules and a few gliosomes The perinodal astrocytic processes gave rise to microvilli-like and some other less delicate processes which passed into the node gap and reached the neighbourhood of the nodal axon membrane (Figs.

21 22, 23 and 24) The nodal extracellular matrix appeared more dense than in the less mature stages but less dense than in the adult (Hildebrand 1971). In some instances an astrocytic cell-body was located in the vicinity of a node. The paranodal myelinoid bodies, corresponding to the light microscopically observed myelin-like bodies, were conspicuous. In contrast to the paranodal myelin sheath outgrowths they were not directly continuous with the myelin lamellae but often seemed to be contained within greatly expanded terminal cytoplasmic pockets (Figs. 23 d and 24).

In general it can be stated that the specialized architecture of nodes in large adult central fibres (Hildebrand 1971) is beginning to appear in the largest fibres of the region studied at the end of the third postnatal week at a fibre size of 4—5 μ.

Later stages. At the end of the first postnatal month the largest fibres measure 6—8 μ (Hildebrand and Skoglund 1971). The nodes of these fibres were qualitatively similar to mature nodes of Ranvier in large central fibres (Hildebrand 1971). The constricted nodal axon was surrounded by a finely granular node gap substance through which several microvilli-like and other processes passed from perinodal astrocytic extensions to the vicinity of the nodal axon membrane (Figs. 25 and 26). On both sides of the node the decrement zones appeared very dense and compact (Fig. 28) containing double piles of small dense terminal cytoplasmic pockets. The pockets in the internodal and nodal ends of the decrement zone were larger and less dense (Figs. 27 and 28). In the internodal end of the decrement zone fingerlike axonal evaginations were present (Fig. 28). The perinodal region was occupied by fibrous astrocytic processes with some gliosomes. Large myelinoid bodies were associated with the paranodal myelin sheaths (Figs. 25 and 27).

The observations on specimens from three month old kittens where the largest fibres measure over 10 μ (Hildebrand and Skoglund 1971) confirmed the findings and did not yield any further details.

Discussion

A large general extracellular space in the immature CNS as compared to the adult has been demonstrated ultrastructurally (Del Cerro *et al.* 1968, Bondareff and Pysh 1968, Pysh 1969, Sumi 1969) and with physiological methods (Vernadakis and Woodbury 1965). Barlow *et al.* (1961) stated that the extracellular sulphate space in the cortex and white matter of the newborn kitten amounts to about 16 and 33 % respectively as compared to an all over size

of 2—4 % in the adult. Thus the large perinodal extracellular space in the immature animals is presumably not an artefact. The extracellular nodal matrix was very scanty in the youngest animals as compared with that of adult cats (Conradi and Skoglund 1969) This seems compatible with reports demonstrating an increased affinity for PAS-staining in the "ground substance" of the developing brain with increasing age (Hess 1955 Urman and Rumley 1958)

It is noteworthy that throughout the postnatal maturation, during which the largest fibres of the region studied increase in size from 2—3 μ to 14—17 μ (Hildebrand and Skoglund 1971) the longitudinal dimension of the decrement zone and the uncovered nodal axon remain at around 4 and 1 μ respectively (cf Hildebrand 1971 Conradi and Skoglund 1969) In the earliest stages, when the number of myelin lamellae and consequently the number of terminal cytoplasmic pockets was small this, as it seemed was due to the comparatively large size and length of apposition of each pocket and also to the presence of cytoplasmic portions continuing the row of terminal cytoplasmic pockets. In more mature stages when the number of myelin lamellae and terminal cytoplasmic pockets was larger all pockets did not appose the axon (cf Hildebrand 1971) Similar dimensions were reported for the decrement zone and the uncovered nodal axon in nodes of adult and developing peripheral nerves (Berthold 1968 a, b) Simultaneously the postnatal increase in diameter of the axon was less nodally than internodally due to the gradually more pronounced axonal constriction in the nodal region with increasing fibre size. Thus the surface area of the uncovered nodal axon and of the part of axon apposed by terminal cytoplasmic pockets does not increase in proportion to the growth in fibre size. From what has been stated above it seems likely that the dimensions of the uncovered nodal axon and the decrement zone are of particular importance (Hess and Young 1952)

In developing peripheral fibres the nodal paranodal region attains a mature ultrastructural and histochemical appearance during growth from 3—6 μ (Berthold 1968 a, Berthold and Skoglund 1967 1968 a, b) Obviously the correlation between fibre size and nodal structural maturation in developing nodes of future large central fibres is similar to that found in the peripheral nervous system. In the peripheral nerves the structural maturation could be correlated to the development of function. Thus at a size of 4 μ the peripheral nerve fibres exhibit nearly adult electrophysiological properties (for references see Skoglund 1966) and fibres around 6 μ behave as in the adult (Ekholm 1967)

Studies on the functional maturation of central fibres are rare. It is known that immature pyramidal fibres have a long latency a long spike duration and

a very limited capacity of transmitting impulses at high frequencies (Purpura et al 1964 Conway et al 1969 Huttenlocher 1970) Similarly the conduction time of afferent central fibres is longer in immature animals than in adults The shorter length of the afferent pathway as compared to the adult does not compensate for the lower conduction velocity (Scherrer 1967) According to Purpura et al (1964) the conduction velocity of feline pyramidal fibres increases greatly during the 4th and 5th postnatal weeks. Simultaneously some fibres become able to maintain sustained activity (Huttenlocher 1970) By the end of the first month conduction velocities of 1 —20 m/sec were recorded and the pyramidal tract was electrographically similar to that in adult animals (Purpura et al 1964) Concurrently the dendritic pattern of the large pyramidal neurons attained an adult appearance During the 4th and 5th postnatal weeks the largest feline pyramidal fibres grow in size from 3 to 5 microns (Hildebrand and Skoglund 1971) Apparently the period of major functional improvements of the pyramidal fibres coincides with the structural maturation of the nodes of Ranvier provided that the present results are valid for pyramidal fibres.

The largest fibres in the superficial part of the spinal cord lateral funiculus which were studied in this report most likely belong to the spinocerebellar tracts (Grant 1962) That the large fibres of two areas with a location corresponding to the dorsal and ventral spinocerebellar tracts respectively increase in size equally much during the postnatal period, measuring 4—5 μ at three weeks and well above 6 μ (7—9 μ) by six weeks was demonstrated earlier (Hildebrand and Skoglund 1971) Since in the kittens decerebrate rigidity does not appear in the hindlimbs until after three weeks (Skoglund 1960) and since the cerebellum seems to be involved in decerebrate rigidity (Terzuolo and Terzian 1953) it appears that the spinocerebellar fibres in analogy with pyramidal fibres and peripheral nerve fibres begin to function efficiently when they reach a size of 4—5 At this size the nodes of Ranvier attain a close to mature morphology

In addition the neurons in Clarke s column (Smith 1969) and to some extent also the cerebellar Purkinje cells (Purpura et al 1964) present a basically mature pattern in three week old kittens. Skoglund and Romero (1965) found that most of the future large feline dorsal root hindlimb afferents do not exceed 3 microns until after 20 days. The latter would mean that after this age the peripheral muscle spindle afferents from the kitten s hindlimb have reached close to adult functional properties. It is known that a number of these fibres have synaptic contact with neurons in Clarke s column (see Laporte et al 1956) Thus the maturation of each component in this afferent pathway fits into a common pattern the whole system being nearly mature by the age of

3 weeks. At this age the kittens are able to stand and walk on the hindlimbs.

At the level of the seventh lumbar segment in the dorsal columns the largest fibres, which enter the spinal cord at sacral levels, reach a size around 5 μ during the second postnatal week. The extensions of the same fibres in the gracile fasciculus at the first cervical segment do not achieve that size until the sixth week (Hildebrand and Skoglund 1971) This might imply that different segments of the same fibre become structurally and possibly also functionally mature at different ages. Ekholm (1967) found that the maximal spike frequency of touch and hair units mediated by large sural nerve fibres increased with increasing age up to six weeks. In six week old kittens the fastest sural nerve fibres conducted at 30—35 m/sec (Ekholm 1967) corresponding to a fibre size around 5.5—6.5 μ (Hursh 1939 Ekholm 1967) Ekholm (1967) thought that the functional changes mentioned were associated with the properties of the afferent fibre rather than with the receptor itself Since some large sural nerve fibres from touch and hair receptors will enter the spinal cord with the first sacral dorsal roots and project to the gracile nuclei (Petit and Burgess 1968 Burgess *et al.* 1968) it seems that the terminal *central and peripheral extensions of these dorsal root fibres follow a similar* developmental time schedule.

The evidence presented here and the discussion above support the idea that the nodal morphology and function of developing future large central fibres become close to mature at 4—5 μ If this can be applied as a general rule new possibilities appear for the research in the field of nervous development, since then it would be possible to evaluate the functional stage of future large fibres in central fibre tracts by measuring their diameters. This would for example be of great value in comparative studies on the development of the central nervous system in man (cf Nyström and Skoglund 1965 Hildebrand and Skoglund 1967)

Summary

The morphological maturation of the nodes of Ranvier in future large fibres in the superficial part of the feline spinal cord lateral funiculus at the first cervical segment was studied with the light and electron microscope The animals were fixed by perfusion with glutaraldehyde. In 2 μ fibres (newborn stage) the nodal structure was very simple and in many respects resembled the pattern in nodes of similarly sized adult central fibres. Concomitantly with the postnatal growth in fibre size the nodes gradually became more complex and at a diameter of 4—5 μ (three weeks) the characteristics of the adult node began to appear At a fibre size of 5—6 μ or more the nodes of Ranvier were

qualitatively similar to nodes of large adult fibres. The findings were correlated with fibre size measurements and physiological data on developing central fibres and some comparisons with the development of peripheral nerve fibres were made

Acknowledgements

This investigation was supported by grants from Karolinska Institutet (Reservationsanslaget) and from the Swedish Medical Research Council (projects no. B70-14X 136-06 and B71 14X-136-07A) I am much indebted to Professor Sten Skoglund for his encouragement and constructive criticism and to Mrs. Ingegerd Lindqvist for excellent technical assistance.

References

Allerand C. D and Murray M. R. Myelin formation in vitro. Endogeneous influences on cultures of newborn mouse cerebellum. *Arch. Neurol.* 19 292—301 (1968)

Barlow C. F Domek, N S Goldberg M. A. and Roth L. J Extracellular brain space measured by S^{35} sulphate *Arch. Neurol.* 5 102—110 (1961)

Bensted J P M, Dobbing J Morgan R. S., Reid R. T W and Wright G. P Neuroglial development and myelination in the spinal cord of the chick embryo. *J Embryol. Exp Morph.* 5 428—437 (1957)

Berthold C. H. Ultrastructure of postnatally developing peripheral nodes of Ranvier *Acta Soc Med Upsal.* 73 145—168 (1968)

Berthold C.-H. Ultrastructure of the node-paranode region of mature feline ventral lumbar spinal-root fibres. *Acta Soc Med Upsal.* 73 Suppl. 9 37—78 (1968 b)

Berthold, C.-H. A study of the fixation of large mature feline myelinated ventral lumbar spinal-root fibres. *Acta Soc Med Upsal.* 73 Suppl. 9 1—36 (1968 c)

Berthold C.-H. and Skoglund, S. Ultrastructure and histochemistry of the developing node of Ranvier in the hindlimb nerves of the cat. *Acta Soc Med Upsal.* 70 287—293 (1965)

Berthold C. H. and Skoglund, S. Histochemical and ultrastructural demonstration of mitochondria in the paranodal region of developing feline spinal roots and nerves. *Acta Soc Med Upsal.* 72 37—70 (1967)

Berthold C.-H. and Skoglund S. Postnatal development of feline paranodal myelin-sheath segments. I. Light microscopy *Acta Soc Med Upsal.* 73 113—126 (1968 a)

Berthold C. H. and Skoglund S. Postnatal development of feline paranodal myelin-sheath segments. II. Electron microscopy *Acta Soc Med Upsal.* 73 127—144 (1968 b)

Bondareff W and Pysh, J J Distribution of the extracellular space during postnatal maturation of rat cerebral cortex *Anat. Rec.* 160 773—780 (1968)

Bornstein M. B. and Murray M. R. Serial observations on patterns of growth, myelin formation, maintenance and degeneration in cultures of new-born rat and kitten cerebellum. *J Biophys. Biochem Cytol* 4 499—504 (1958)

BOYDE, A., JAMES D W TRESMAN R. L. and WILLIS R. A. Outgrowth from chick embry spinal cord studied w th the scanning electron microscope *Z Zellforsch 90* 1—18 (1968)

BRODY I The keratinization of epidermal cells of normal guinea pig skin as revealed by electron microscopy *J Ultrastruct. Res 2* 48?—511 (1959)

BUNGE, R. P Glial cells and the central myelin sheath. *Physiol. Rev 48* 197—.51 (1968)

BUNGE, M B., BUNGE, R. P and PAPPAS, G D Electron microscopic demonstration of connections between glia and myelin sheaths in th developing mammalian central nervous system. *J Cell Biol. 12* 448—453 (1962)

BUNGE, M. B., BUNGE, R. P and RIS, H Ultrastructural study of remy lination in an experimental lesion in adult cat spinal cord *J Bi phys Biochem. Cytol. 10* 67—91 (1961)

BURGESS, P R., PETIT D and WARREN R. M. Receptor types in cat hairy skin supplied by myelinated fibres. *J Neurophysiol. 31* 833—848 (1968)

CONRADI, S Observations on the ultrastructure of the axon hillock and initial axon segment of lumbosacral motoneurons in the cat. *Act Physiol. Scand* Suppl. 322, 65—84 (1969)

CONRADI, S. and SKOGLUND S. Observations on the ultrastruct re of the initial motoraxon segment and dorsal root boutons on the motoneurons in the lumbosacral spinal cord of the cat during postnatal development. *Acta Physiol. S and* Suppl. 333 53—76 (1969)

CONW C. J WRIGHT F S and BRADLEY W E. Electrophysiological maturation of the pyramidal tract in the postnatal rabbit. *Elect oenceph Clin. Neurophysiol. 26* 565—577 (1969)

DEL CERRO, M. P SNIDER, R. S. and OSTER, M. L. E volution of the extracell lar space in immature nervous tissue. *Experientia. 24* 929—930 (1968)

DE ROBERTIS, E., GERSCHENFELD, H. and WALD, F Cellular mechanism of myelination in the central nervous system. *J Bi phys Biochem Cytol. 4* 651—658 (1958)

EKHOLM J Postnatal changes in cutaneous reflexes and in the discharge pattern of cutaneous and articalur sense organs. *Acta Physiol. S and* Suppl. 297 1—124 (1967)

FIELD E. J HUGHES, D and RAINE C. S Electron microscopic observations on the de velopment of myelin in cultures of neonatal rat cerebellum. *J Neurol. Sci. 8* 49—60 (1969)

FLEISCHHAUER, K. Postnatale Entwicklung der Neuroglia. *Act Neuropath* (Berlin) Suppl. 4 ?0—32 (1968)

FLEISCHHAUER, K and WARTENBERG H. Elektronenmikroskopische U tersuchungen über das Wachstum der Nervenfasern und über das Auftreten von Markscheiden im Corpus callosum der Katze. *Z Zellforsch 83* 568—581 (1967)

FRIEDE, R. L. and SAMORAJSKI, T Relation between the number of myelin lamellae and axon circumference in fibres of vagus and sciatic nerves of mice. *J Comp Neurol 130* 223—232 (1967)

GALEY F R and NILSSON S E. A new method for transferring sections from the liquid surface of the trough through staining solutions t the supporting film of the grid. *J Ultrastru t R 14* 405—410 (1966)

GEREN-UZMAN, B B The spiral configuration of myelin lamellae. *J Ultrastruct Res 11* 208—212 (1964)

GRAY G Spinal course and somatotopically localized termination of th spinocerebellar

tracts. An experimental study in the cat. *Acta Physiol. S nd* 56 Suppl. 193 1—43 (1962)
HASHIMOTO P H. Electron microscopic study of gliosome formation in postnatal development of the spinal cord in the cat. *J Comp Neurol* 137 251—255 (1969)
HESS, A. Postnatal development and maturation of the nerve fibres of the central nervous system. *J Comp Neurol* 100 461—480 (1954)
HESS, A. Blood-brain barrier and ground-substance of central nervous system. Similarities in development. *Arch. Neurol. Psychiat* 73 380—385 (1955)
HESS, A. and YOUNG, J Z. The nodes of Ranvier *Proc Roy Soc B.* 140 301—320 (195)
HETZKO D Über die postnatale Zunahme des Capillarvolumens im Corpus callosum der Katze. *Z. Anat. Entwickl ngsgesch* 127 138—144 (1968)
HILD, W. Myelogenesis in cultures of mammalian central nervous tissue *Z Zellforsch* 46 71—95 (1957)
HILDEBRAND, C. Ultrastructural and light-microscopic studies of the nodal region in large myelinated fibres of the adult feline spinal cord white matter *Act Physiol. Scand* Suppl. 364 43—80 (1971)
HILDEBRAND C. and SKOGLUND, S. Ultrastructural features of the nodal region in lumbar spinal roots of newborn man. *Act Soc Med. Upsal.* 7 71—75 (1967)
HILDEBRAND C. and SKOGLUND S Calibre spectra of some fibre tracts in the feline central nervous system during postnatal development. *Acta Physiol. Scand* Suppl. 364 5—4 (1971)
HILLEBRAND H. Quantitative Untersuchungen über postnatale Veränderungen der Glia im Corpus callosum der Katze *Z Zellfor h.* 5 303—312 (1966)
HIRANO, A., LEVINE, S and ZIMMERMAN H. M. Remyelination in the central nervous system after cyanide intoxication. *J Neu path Exp. Neurol* 77 234—245 (1968)
HODGKIN A. L. The ionic basis of electrical activity in nerves and muscle. *Biol Reviews* 26 339—409 (1951)
HUXLEY A. F and STÄMPFLI, R. Evidence of saltatory conduction in peripheral myelinated nerve fibres. *J Physiol* (London) 108 315—339 (1949)
HURSH, J B. Conduction velocity and diameter of nerve fibers. *Amer J Phy iol.* 127 131—139 (1939)
HUTTENLOCHER, P R. Myelination and development of function in immature pyramidal tract. *E p Neurol.* 29 405—415 (1970)
KENNEDY C., GRAVE, G D JEHLE, J W and SOKOLOFF L. Blood flow to white matter during maturation of the brain. *Neu ology* (Minneap.) 20 613—618 (1970)
LANDON, R. H. and COWAN W M A structural specialization at nodes of Ranvier in the central nervous system. *N t* 210 757—758 (1966)
LAMPERT P Demyelination and remyelination in experimental allergic encephalomyelitis. Further electron microscopic observations. *J Neuropath. Exp Neurol* 24 371—384 (1965)
LANDON D N and WILLIAMS P L. Ultrastructure of the node of Ranvier *Nature* 199 575—577 (1963)
LAPORTE, Y LUNDBERG, A and OSCARSSON O Functional organization of the dorsal spinocerebellar tract in the cat. *Acta Physiol S and.* 36 188—203 (1956)
LUSE, S A. Formation of myelin in the central nervous system of mice and rats as studied with the electron microscope *J Biophys. Biochem Cytol.* 2 777—784 (1956)

LUSE, S A. Fine structure of the morphogenesis of myelin. pp. 59—95 in *The Biology of Myelin* (S. Korey ed) Hoeber Harper New York (1959)

MAUNSBACH, A. B., MADDEN S C. and LATTA, H. Variations in fine structure of renal tubular epithelium under diff rent conditions of fixation. *J Ultrastruct Res. 6* 511—530 (1962)

MILLONIG, G Advantages of a phosphate buffer for OsO solutions in fixation. *J Appl. Physics 32* 1637 (1961)

NYSTRÖM B. and SKOGLUND, S Calibre spectra of spinal nerves and roots in newborn man. *Act M ph Neerl.-Scand. 6* 115—127 (1965)

OSCARSSON O and UDDENBERG N Identification of a spinocerebellar tract activated from forelimb afferents in the cat. *Acta Physiol. Sca d 62* 125—136 (1964)

PALADE, G E. A study of fixation for electron microscopy *J Exp. Med 95* 285—297 (1952)

PETERS A. The formation and structure of myelin sheaths in the central nervous system. *J Biophys Biochem. Cytol. 8* 431—446 (1960)

PETERS A. Further observations on the structure of myelin sheaths in the central nervous system. *J Cell Biol. 20* 281—296 (1964)

PETERS, A. The nodes of Ranvier in the central nervous system. *Quart J Exp Physiol. 51* 229—236 (1966)

PETIT D and BURGESS, P R. Dorsal column projection of receptors in cat hairy skin supplied by myelinated fibres. *J Neur physiol. 31* 849—855 (1968)

PURPURA, D P SHOFER, R. J HOUSEPIAN E. M. and NO ACK, C. R. Comparative ontogenesis of structure-function relations in cerebral and cerebellar cortex. pp. 187—221 in *P gres i Brai Research 4* (D P Purpura and V P Schadé eds.) Elsevier Amsterdam (1964)

PYSH J J The development of the extracellular space in neonatal rat inferior colliculus. An electron microscopic study *Amer J Anat 124* 411—430 (1969)

REYNOLDS, E. The use of lead citrate at high pH as an electron opaque stain in electron microscopy *J Cell. Biol. 17* 208—212 (1963)

ROSS, L. L., BORNSTEIN M. B. and LEHRER, G M. Electron microscopic observations on myelin sheath formation in tissue cultures of developing rat cerebellum. *Anat Rec 136* 268 (1960)

ROSS, L. L BORNSTEIN M B. and LEHRER, G. M. Electron microscopic observations of rat and mouse cerebellum in tissue culture. *J Cell Biol. 14* 19—30 (196.)

RYTER, A. and KELLENBERGER, E. L'inclusion au polyester pou l'ultramicrotomie *J Ultrastruct Res. 2* 200—214 (1958)

SAMORAJSKI, T and FRIEDE, R. L. A quantitative electron microscopic study of myelination in the pyramidal tract of rat. *J Comp Neurol. 134* 323—338 (1968)

SCHERRER, J Electrophysiological aspects of cortical development. pp. 480—489 in *Pr gress in Brain Res h 22* (E. A. Asratyan ed.) Elsevier Amsterdam (1967)

SKOGLUND S On th postnatal development of postural mechanisms as revealed by electromyography and myography in decerebrate kittens. *Acta Physiol. Scand 49* 299—317 (1960)

SKOGLUND S Muscle afferents and motor control in the kitten. pp. 245—259 in *Muscular Afferents and M tor C trol*, Nobel Symposium I (R. Granit ed.) Almqvist & Wiksell, Stockholm (1966)

SKOGLUND, S and R MERO C. Postnatal growth of spinal nerves and roots. *Act Physiol. S and 66* Suppl. 260 1—50 (1965)

SMITH, D. E. Observations on the postnatal development of Clarke column in the kitten. *J Comp Neurol. 135* 263—74 (1969)
STORTS, R. W. and KOESTNER, A. Development and characterization of myelin in tissue culture of canine cerebellum. *Z. Zellforsch. 95* 9—18 (1969)
STM, S. M. The extracellular space in the developing rat brain. Its variation with changes in osmolality of the fixative, methods of fixation and maturation. *J Ult ... Res* 29 398—4 5 (1969)
TASAKI, I. The electro-saltatory transmission of the nerve impulse and the effect of narcosis upon the nerve fiber. *Amer J Physiol 127* 11—227 (1939)
TERZUOLO C. and TERASIAN H. Cerebellar increase of postural tonus after deafferentation and labyrinthectomy. *J Neurophysiol. 16* 551—561 (1953)
UZMAN, L. L. and RUMLEY M. K. Changes in the composition of the developing mouse brain during early myelination. *J Neurochem. 3* 170—184 1958)
VAUGHN J. E. An electron microscopic analysis of gliogenesis in rat optic nerve. *Z Zellforsch. 91* 293—324 (1969)
VAUGHN J. E. and PETERS, A. Electron microscopy of the early postnatal development of fibrous astrocytes. *Amer J Anat 121* 131—15 (1967
VERNADAKIS A. and WOODBURY D. M. Cellular and extracellular spaces in developing rat brain. *A. A. Neurol. 12* 284—293 (1965)
WECHSLER, W. Elektronenmikroskopischer Beitrag zur Histogenese der weissen Substanz des Rückenmarks von Hühnerembryonen. *Z. Zellforsch. 74* 23 —251 (1966)
WILLIAMS P. L. and LANDON D. N. Paranodal apparatus of peripheral myelinated nerve fibres of mammals. *Nature 198* 670—673 (1963)

Legends to figures

Figs. 1—7 are light micrographs and Figs. 8—28 electron micrographs.

Fig. 1 Newborn kitten. Longitudinal section. The picture shows two nodes (arrows) The nodal axons are slightly constricted and the axoplasm has a low density The decrement zones appear as diffusely outlined cuffs which are less dense than the myelin. Note the undulant course of the fibres. (1500 ×)

Fig. 2. Newborn kitten. Node of Ranvier (arrow) as in Fig. 1 Not bow the myelin sheaths turn out from the axon on both ides of the node. (1500 ×)

Fig 3 Two week old kitten. Longitudinal section. Node of large fibre (arrow) A dense nodal axoplasm, compact decrement zones and a distinct constriction of the axon can be seen. (B = blood vessel) (1500 ×)

Fig 4 Three week old kitten. Longitudinal section. Node of large fibre (arrow) with a dense constricted nodal axon and compact decrement zones. (1500 ×)

Fig 5 Four week old kitten. Longitudinally sectioned node (arrow) of large fibre (about 6 μ) The constriction of the nodal axon and the density of the decrement zones is more pronounced. The myelin sheaths turn in towards the axon on both sides of the node (1500 ×)

Fig. 6. Three month old kitten. Longitudinal section. Node of large fibre (arrow) Note the axoplasmic density the axoplasmic granules the constriction of the nodal axon and the dense appearance of the decrement zones. The node gap appears as narrow slot between the inturning myelin sheaths. (1500 ×)

Fig 7 a—e. Two week old kitten. Series of transverse sections through nodal paranodal region of a large fibre (axon indicated by a, B = blood vessel) Fig 7 a. First section, about 6 μ before the node. Note the compact myelin-like bodies (indicated by x) outside the myelin sheath. Fig. 7 b. Nine sections later The plane of section goes through the decrement zone The axon diameter has become reduced. Note the astrocyte in the vicinity (N = nucleus) Fig. 7 c. Section number 1 through the nodal end of the decrement zone. Fig 7 d. Next section. The plane of section goes through the node gap. The nodal axon is nearly invisible. The surroundings are occupied by pale cytoplasm. (N = astrocytic nucleus) Fig 7 e. Seven sections later the fibre has returned to larger diameter (2000 ×)

Fig. 8. Newborn kitten. Longitudinal section through node of Ranvier The axon (A) is constricted in the decrement zones and bulges out in the node gap (NG) The node gap appears nearly empty only containing small amounts of a flocculent material. All terminal cytoplasmic pockets ppose the axon. Note the low electron-density of the pocket cytoplasm. The right decrement zone is continued in nodal direction by cytoplasmic portions (arrows) which are not continuous with myelin lamellae. (15,200 ×)

Fig 9 Detail of Fig. 8 showing the nodal end of upper right decrement zone. (A = axon, NG = node gap) The nodal axon membrane is coated on its axoplasmic side by a dense material (small arrows) The undercoating is absent where the axon is apposed by cytoplasmic portions (large arrows) (60,800 ×)

Fig 10. Four day old kitten. Longitudinal section. Detail of decrement zone. The myelin lamellae successively end as terminal cytoplasmic pockets which ppose the axon (A) Between adjacent pockets desmosome-like junctions are seen (arrows) (52,900 ×)

Fig. 11. Four day old kitten. Transverse section through decrement zone. The axon (A) is circular in outline and surrounded by the uncoiling lateral cytoplasmic borde of the myelin sheath (MS). Within this cytoplasm a mitochondrion (m) can be seen. (15 400 ×)

Fig. 12. Newborn kitten. Transverse section through node of Ranvier. The uncovered nodal axon (A) is surrounded by a fairly extensive extracellular space containing small amounts of a flocculent material. Note the irregular contou of the axon and the absence of any specific astrocytic processes such as in the mature node. (21,000 ×)
Inset shows the nodal axon membrane of the same fibre at higher magnification (A = axon). The axoplasmic side of the membrane is coated by dense material (arrows). (69,300 ×)

Fig. 13. Newborn kitten. Longitudinal tangential section through node of Ranvier. (A = axon, NG = node gap). Both decrement zones are ontinued in nodal direction by cytoplasmic portions (large arrowheads). Small arrows indicate continuity between cytoplasmic portion and the cytoplasm of the myelin sheath. (18,900 ×)

Fig. 14. Newborn kitten. Longitudinal section. Decrement zone with two highly enlarged terminal cytoplasmic pockets containing dense lamellar and granular inclusions (arrows). The other terminal cytoplasmic pockets ppear normal. (A = axon, NG = node gap). (22,000 ×)

Fig. 15. Newborn kitten. T ansverse section through decrement zone. The cytoplasm of the terminating myelin sheath (MS) forms an irregular collar around the nearly circular axon (A). Note the two my lin-like bodies (arrows). (12,650 ×)

Fig. 16. Four day old kitten. Longitudinal section. Two nodal regions are seen (nodal axons indicated by A). Note the axonal irregularities in the decrement zones (small arrows) and the partly detached terminal cytoplasmic pockets (large arrows). (9000 ×)

Fig. 17. Four day old kitten. Longitudinal section through decrement zone. Note how the four most nodal terminal cytoplasmic pockets (indicated by 1, 2, 3 and 4) on each side of the axon (A) are peeled off by extensions from adjacent pockets (small arrows). Large arrow points in nodal direction. (29 400 ×)

Fig. 18. One week old kitten. Longitudinal section. The picture shows a closed node of Ranvier (A = axon). Note how the decrement zone of the left myelin sheath continues outside the opposite myelin sheath and ends to the right in the picture (large arrow). Small arrows indicate the end of the right myelin sheath. (10,000 ×)

Fig. 19. One week old kitten. Longitudinal section. At place corresponding to the node the axon (A) is expanded and filled with arious lamellated bodies. The node is closed by extensions from the decrement zones (small arrows). (12,200 ×)

Fig. 20. Tw week old kitten. Longitudinal section through node of Ranvier. The axon (A) contains several vesicles, some dense bodies and a few elongated mitochondria (m). Note the cytoplasmic portions and the varying size and shape of the terminal cytoplasmic pockets. In the node gap several slender astrocytic extensions can be seen. These arise from larger perinodal astrocytic processes (PAP) which contain filaments and a gliosome (G). The nodal extracellular space is rather extensive. (11 700 ×)

Fig. 21. Three week old kitten. Longitudinal section. Nodal region of large fibre. The axon (A) is learly constricted in the decrement zones and bulges out in the node gap.

The axoplasm contains several vesicles some dense bodies and a few elongated mitochondria (m) Large arrow indicates axonal evaginations in the internodal end of the decrement zone. All terminal cytoplasmic pockets do not appose the axon. The perinodal region is filled with fibrous astrocytic processes (PAP) and the extracellular space of the node gap is reduced. (8400 ×)

Fig. 22. Three week old kitten. Tangential longitudinal section of the nodal region shown in Fig. 22. The decrement zones are indicated by DZ. A large fibrous astrocytic process (AP) is directed towards the node. Note the elongated gliosome (G) In the node gap a tangentially cut outbulging (a) of the nodal axon and astrocytic cytoplasm (large arrows) can be seen (11,300 ×)

Fig. 23 a, b c and d. Three week old kitten. Series of transverse sections through node of Ranvier Fig. 23 a. The nodal axon (A) contains vesicles, some dense bodies and several closely spaced neurotubules and neurofilaments. The axon is partly surrounded by cytoplasm of the proximal decrement zone (DZ) In the upper left part of the picture the axon shows an outgrowth containing a dense body Outside the outgrowth astrocytic processes (AP) can ben seen. One of these emits a few microvilli-like processes (small arrows) which pass towards the nodal axon membrane. Fig 23 b. Two sections later The axonal outgrowth has increased in size and greater part of the axon (A) is uncovered. Note the perinodal astrocytic processes (AP) and the microvilli-like processes (small arrows) Fig 23 c. Next section. A = axon, AP indicates perinodal astrocytic processes and small arrows point to microvilli-lik processes. Fig. 23 d. Several sections later The plane of section goes through the distal decrement zone. Note the nearly circular contour of the axon (A) which is covered by the ter minating myelin sheath (DZ) outside which two myelinoid bodies can be seen (large arrows) a, b and (23,800 ×) d (10,900 ×)

Fig 24 Three week old kitten. Longitudinal section passing tangentially through a nodal region. A = axon, DZ = decrement zones. In the node gap (small arrows) the tangentially cut nodal axon (a) is apposed by delicate processes. Perinodal astrocytic processes with some gliosomes (g) can be seen. Th right decrement zone shows a large outgrowth (large arrow) and also encloses myelinoid body (MB) Note the myelin sheath outgrowth in th left paranod (large arrow) and the irregularities of the axon in the internodal end of the left decrement zone (small arrows) (7800 ×)

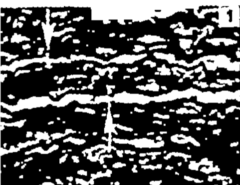
Fig 25. Four week old kitten. Longitudinal section. Nodal region of large fibre. Note the pronounced nodal constriction of the axon (A) and the myelinoid bodies outside th terminating myelin sheaths (large arrows) Perinodal astrocytic processes (PAP) and cytoplasmic extensions in the node gap (small arrows) can be seen. (7100 ×)

Fig. 26 Node gap of fibre shown in Fig. 25 at higher magnification. (A = axon) Arrows point t microvilli-like processes. Outside th nod gap perinodal astrocytic processes (PAP) are seen. Note the finely granular extracellular substance. (12 000 ×)

Fig 27 Five week ld kitten. Longitudinal section. Nodal region of large fibre A branch (small arrow) is given off from the nodal axon (A) Note the perinodal astrocytic cytoplasm (As) containing gliosomes (G) Large arrow indicates paranodal myelinoid body (5600 ×)

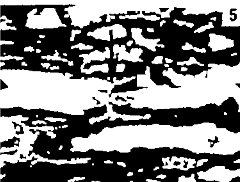
Fig. 28 Six week ld kitten. Longitudinal section. Nodal region of large fibre. The constricted axon (A) contains many vesicles, some dense bodies and few mitochondria (m) Large arrows indicate axonal evaginations in th internodal end of the

left decrement zone Small arrow points t nod gap. Outside the nod gap perinodal astrocytic processes are present (PAP) Note the density of the decrement zones. (7900 ×) Inset shows tangential longitudinal section of internodal end of a decrement zone in a six week old kitten. (A = axon, DZ = cytoplasm of terminating my lin sheath) Note the axonal evaginations (small arrows) (9900 ×)


1

2

3
B

4

5

6

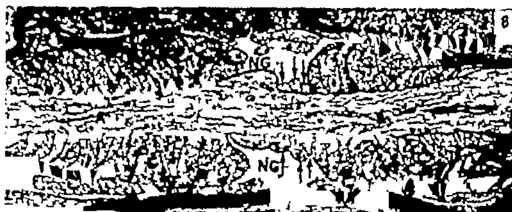
7a
B

7b
N
B

7c
N
B

7d
N
B

7e
B


8
NG
NG


NG
9

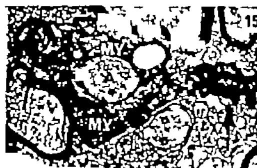

10
A


11


12


NG
A
13


14


15
MY
MY


16


17


18


19


20


21


22




23b
AP


23c
AP
AP
AP


23d
DZ
DZ


24
MB
A
A


25


26


27


28


23a
DZ


23b


23c
AP
AP
AP
A


23d
DZ
DZ


24
MB
A
A

Ultrastructural and light microscopic studies of the developing feline spinal cord white matter II Cell death and myelin sheath disintegration in the early postnatal period

By

Claes Hildebrand

Introduction

It is well known that degeneration and selective cell death is a normal corollary of cellular proliferation and differentiation in vertebrate ontogeny (see reviews by Ernst 1926 and Glücksmann 1951). Degenerative patterns have been observed in the embryonic nervous system (Hamburger 1952, Levi Montalcini 1964, Källén 1965, Hughes *et al.* 1969). In two recent reports by Berthold and Skoglund (1968 a, b) degenerative features, apparently representing a myelin sheath disintegration and segmental demyelination were found to occur as a normal developmental process in early postnatal feline lumbar spinal roots. In the spinal cord white matter of immature postnatal rabbits Spatz (1918) noted degenerating glial cells and according to Mori and Leblond (1970) degenerating oligodendrocytes are present in the corpus callosum of young rats. Furthermore Bodian (1966) has described a spontaneous neuronal degeneration in the spinal cord of the newborn monkey (cf. Wechsler 1966a).

During the progress of studies on the feline central nervous system dealing with the postnatal change in fibre size distribution of some fibre tracts (Hildebrand and Skoglund 1971) and the postnatal structural maturation of the central node of Ranvier (Hildebrand 1971 a) certain odd myelin sheath configurations and a number of simultaneous glial changes, suggestive of a degeneration process, were repeatedly encountered. It is the purpose of the present paper to describe these features as seen with the light and electron microscope. The findings have been interpreted as signs of a selective degeneration of myelin-glial units in the spinal cord white matter during the first postnatal weeks.

Material and methods

Specimens from 15 kittens in ages varying from one day to 3 months were studied. The central nervous system was fixed by vascular perfusion with 5 % glutaraldehyde in a 300 mOsm Millonig buffer containing 2.7 % Rheomacrodex (Berthold 1968 a). Specimens comprising the lateral funiculus of the first cervical spinal cord segment were postfixed, rinsed, postosmicated, acetone dehydrated and embedded in Vestopal W (Ryter and Kellenberger 1958). For methodological details see Hildebrand (1971 b). For light microscopy series of 100—200 transverse and longitudinal semithin (0.5 μ) sections were cut with a LKB 4801 Ultrotome and stained with toluidine blue. The studies were limited to the superficial part of the lateral funiculus. For electron microscopy series of 100—200 consecutive transverse or longitudinal thin sections with a grayish interference colour were cut from the same region. The thin sections were stained with uranyl acetate (Brody 1959) and lead citrate (Reynolds 1963). The series of semithin and thin sections were examined in a Leitz Wetzlar photomicroscope and a Philips EM 300 electron microscope respectively.

For comparative purposes the Wallerian degeneration in the gracile fasciculus resulting from severance of the ipsilateral L6, L7 and S1 dorsal roots was studied in two kittens. The roots were cut at the age of one and two weeks respectively and the postoperative survival time before perfusion was 5 days.

Since it would be of relevance for the discussion to know the postnatal growth in length of the feline spinal cord, measurements were made upon 44 recently killed kittens and adult cats, which had been used for physiological experiments. The distance measured extended from the level of foramen magnum to the lower border of the seventh lumbar spinal cord segment. During the measurements the spine was maintained as straight as possible.

Results

Light microscopy

During the first postnatal weeks numerous glial cells were present in the region studied. Although the appearance of these cells was rather variable most of them could be classified in the light microscope as either oligodendrocytes or astrocytes. The microglia was difficult to recognize light microscopically but its presence was confirmed in the electron microscope (see below). Oligodendrocytes were characterized by their dark blue cytoplasm, the well defined outline and the large round nucleus with a prominent nucleolus.

Often oligodendroglial processes extended to myelinating fibres (cf. Bunge *et al.* 1962). The astrocytes were recognized by their abundant, slightly mottled, pale cytoplasm, the diffuse outline and the irregularly rounded or elongated nucleus. Among the glial cells mitotic figures were rather common. In longitudinal sections the glial cells were often arranged in rows between the myelinating fibres (see Penfield and Oxon 1924).

The myelinating fibres followed an undulant course and many of the myelin sheaths were partly irregular and distorted by multiple outgrowths. Outside the myelin sheaths could be seen some dense globules which were stained like myelin. (Figs. 1, 3 and 4). Similar myelin-like globules were, as it seemed, associated with glial cells which either resembled oligodendrocytes or appeared to be microglia. Some myelinated small fibres showed unmyelinated segments along which dense fragments and what appeared as empty vacuoles occurred. (Fig. 2). The myelin sheath outgrowths and dense myelin-like globules were less prominent during the first than during the second and third postnatal weeks. Large flattened myelin sheath expansions, i.e. redundant myelin (Rosenbluth 1966) and what at first appeared as myelinated oligodendrocytes were common, particularly in kittens less than two weeks old. By following the series of sections it was ascertained that the oligodendroglial myelin covering was incomplete and represented a myelin sheath extension partly wrapped around the glial cell (Fig. 5). Thus the latter feature is a variety of redundant myelin sheath. The axon contained within a redundant myelin sheath was always small.

Certain glial cells with a small dense irregular, U-shaped or triangular nucleus and a moderately dense, sparse cytoplasm were conspicuous. The outstanding feature of these cells was that they contained large vacuoles or inclusion bodies. The inclusions appeared as dense, well delimited rounded bodies of a deep violet or bla k colour. Other similar cells were loaded with small dense fragments. Mixed or intermediate forms also occurred (Figs. 5b, 6 and 7).

Furthermore some structures which consisted of a deep violet dense nucleus surrounded by a less dense m. ginal zone were encountered. The marginal zone varied from being only sl htly less sta ned than the central nucleus to an almost empty appearance. Sometimes the central nucleus was incompletely filled with dense material. The hole nucleated structure had a diameter of 5—10 μ (Figs. 8, 9 and 10). Th question wh her the nucleated bodies were contained within a l al cell or could not e settled with the light microscope.

The inclusion-con ining cells an the nucleated o dies were present throughout the first three stnatal weeks but were mo frequent during the first

meter around 1 μ) in one end and the other sometimes joining an oligodendrocyte. In addition to the small axon a moderately dense glial cytoplasm was contained within the irregular space enclosed by the myelin sheath (Figs. 19 and 20)

The immature myelin sheaths also showed other irregularities. As noted with the light microscope it was common to find large compact myelin-like bodies external to the myelin sheaths, particularly along the larger fibres. These bodies frequently displayed a lamellar substructure similar to that of myelinoid bodies in adult white matter (period 45—50 Å) (see Hildebrand 1971 b) and their lamellae were not continuous with those of the myelin sheath (cf Hildebrand 1971 a) as seen from the serial sections.

The cytoplasmic components of the myelin sheaths such as the inner cytoplasmic tongue or the terminal cytoplasmic pockets, were sometimes focally enlarged. Within the enlarged portions of cytoplasm aggregates of numerous small (diameter 700—900 Å) spherical vesicles were often noted. As a rule the myelin sheaths showing such changes were more or less distorted by multiple outgrowths of varying size and shape Within the outgrowths a glial cytoplasm containing the vesicular aggregates was present (Figs. 16 and 17) In the enlarged cytoplasmic compartments dense polymorphous bodies composed of a granular or lamellar material also occurred. The lamellar pattern of the fragments often resembled that of myelinoid bodies (Fig 18) As reported elsewhere (Hildebrand 1971 a) some nodes of Ranvier showed an abnormal configuration during the first two weeks. Lamellated fragments in what appeared as various stages of disintegration, a dense granular material and vesicular aggregates could also be located outside the distorted myelin sheaths or in between widely separated myelin lamellae (Fig 18)

In longitudinal sections the myelin irregularities occupied a variable stretch of the myelin sheath. In the most pronounced cases the entire myelin sheath was highly irregular and formed an exceptionally short internode (5—20 μ) (Figs. 21 23 24 and 25) At both ends of the short internodes the terminal cytoplasmic pockets appeared small and fairly dense (Fig 21 b) Glial processes, probably of astroglial origin penetrated between the axon and the folded myelin sheath thereby separating the terminal cytoplasmic pockets from the axon (Fig 21 b) and also extended between the individual terminal cytoplasmic pockets. The very short internodes were observed in fibres with an axon diameter around 1 μ On both sides of the short internodes the axon, which always seemed normal was usually uncovered for several microns before the neighbouring myelin sheaths appeared.

In transverse sections axons ensheathed only by a few myelin lamellae or unmyelinated with whorls of highly irregular myelin at some distance from

the axon could be seen (Figs. 22 and 27) The myelin loops were inter connected by a fairly dense partly vacuolized cytoplasm which sometimes extended to appose the unmyelinated or poorly myelinated axon (Fig 27) Compact balls of folded myelin such as in Fig 26 which displayed a normal myelin period but lacked any obvious relationship to an axon were also seen, sometimes in association with oligodendroglia.

Unmyelinated axons surrounded by discontinuous myelin fragments partly contained within portions of a fairly dense glial cytoplasm were encountered also in longitudinal sections (Fig 29) Sometimes long unmyelinated axon segments were intercalated between two myelin sheaths of normal apperance. In one case the length of such an unmyelinated segment was 25 μ and in another the uncovered segment measured 5 μ

The microglial cells With respect to microglia the terminology is somewhat confused (see Del Río-Hortega 1932, Russell 1962, Vaughn *et al* 1970) In the description below the term microglia is applied to cells with phagocytic activity and certain structural features in common which distinguish them from oligodendroglia and astroglia, regardless of their possible embryologial origin.

This glial variety occurred either as perivascular cells between the peri vascular basement membranes or as interstitial microglia within the nervous tissue (Mori and Leblond 1969) The perivascular variety had a highly variable shape and the dense nucleus was irregular or lobulated. The Golgi apparatus was prominent and several small dense cored vesicles (diameter 0.1—0.2 μ) were noted. Furthermore there were groups of rounded cytoplasmic bodies with a diameter ranging between 0.5 and 1.5 μ (Fig 28) The cytoplasmic bodies were bounded by a distinct triple-layered membrane the inner leaflet of which was thicker than the outer (Fig 28) The matrix of the cytoplasmic bodies was either (1) dark and finely granular with occa sional light patches or (2) less dark and more coarsely granular or (3) composed of an irregularly distributed flocculent material. In case of the two former varieties an electron translucent zone often separated the matrix from the limiting membrane (Fig 28) Cytoplasmic bodies of the third type usually had irregular contours in contrast to be smooth rounded outline of the other varieties. Due to the structural characteristics these cytoplasmic bodies might represent lysosomes (cf Maunsbach 1966) In addition the microglial cells often contained one or a few larger cytoplasmic bodies (approximate size 1.5—2.5 μ) with a similar limiting membrane as that of the smaller cytoplasmic bodies. The shape and internal structure of these larger bodies varied. Numerous coated vesicles some microtubules, a cen-

triolar complex, a sparse endoplasmic reticulum and a moderate number of elongated mitochondria with distinct cristae were other regular constituents of this cell. Cells with similar features were also present within the nervous tissue. These cells which formed part of the interstitial microglial population, gave rise to long tapering processes In a few instances a microglial cell occupied a position close to an axon surrounded by myelin fragments (Figs. 29 and 30) and seemed to embrace some of the fragments (Fig. 30)

With the light microscope glial cells containing large inclusions were noted in the immature white matter In the electron microscope the microglial nature of the inclusion-containing cells was recognized (cf Bodian 1966) The inclusions presented a highly polymorphous picture and were structurally quite different from the microglial cytoplasmic bodies mentioned above (Figs. 31—35) Nevertheless an attempt was made to classify the microglial inclusions into a few distinct groups on the basis of their structure.

I Some inclusions were made up of what appeared as a fairly compact body of folded myelin membranes (Fig 31) A closer examination revealed that sometimes the alternating light and dense lines were of a uniform thickness and the period was about 45—50 Å. This lamellar pattern is similar to that of myelinoid bodies (see above and Hildebrand 1971 b) In other instances the lamellae displayed a typical myelin structure. It is likely that this kind of *inclusion possibly representing phagocytized myelin (see Gonatas et al 1965)* corresponds to the black inclusions observed in the light microscope.

II Certain inclusions appeared to consist of a fairly dense vesiculated cytoplasm with swollen mitochondria. In these instances the picture was complicated since the microglial cytoplasm protruded into the inclusion subdividing it into more or less irregular compartments (Figs. 32 and 33)

III In other cases the inclusion was composed of a spherical central body with a dense finely granular substructure surrounded by a marginal zone of a more irregularly distributed dense material. The width of the marginal zone varied and it was separated from the central body by a membrane. Rounded delimited corpuscles with a diffuse interior could often be distinguished in the marginal zone (Figs. 34 and 35) Sometimes the dense matrix of the central body was partly defective leaving empty patches. Inclusions with an almost empty central body surrounded by a light marginal zone were a less common variety Here aggregates of a dense material could be located outside the marginal zone.

IV Within some microglial cells masses of an irregularly distributed granular vesicular and lamellated material in varying proportions filled large areas (Fig 35) Among the irreguar constituents in this type of inclusion rounded delimited bodies, some of which resembled mitochondria could sometimes be distinguished. In other instances inclusions of this type contained very little material appearing more like vacuoles.

Possibly the inclusions of type III correspond to those bodies which in the light microscope were dense and had a deep violet colour whereas type IV fits better with the fragmented or vacuolated varieties. The type II inclusions are certainly not light microscopically visible Each of the different inclusions listed above could occur singly or in combination with any of the other varieties within one microglial cell and intermediate forms were also seen. Often the inclusion-containing microglial cells formed small groups of two or three.

Later stages

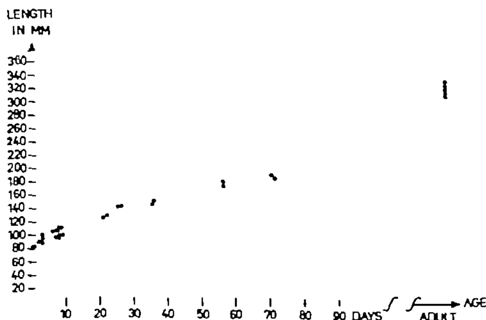
During the third postnatal week the myelinated fibres rapidly increase in number and the tissue acquires a more compact general structure. Six weeks after birth the proportion of glial cytoplasm has become reduced and some fibres are rather large and surrounded by thick sheaths. At three months the general appearance is essentially as in the adult (Hildebrand and Skoglund 1971)

In older kittens the pronounced myelin sheath irregularities and short inter nodes described above were never seen although myelinoid bodies were common. Likewise degenerating oligodendrocytes and except type I inclusion containing microglial cells were not encountered after the third week. Microglial inclusions of type I were in a few instances seen in older animals. The other microglial cells, not containing inclusions, remained infrequent in older kittens as compared to the first postnatal fortnight

Operated animals

The purpose of this section is to point out the similarities between certain of the features described in the preceding text, which occurred in normal white matter during the early postnatal period, and some of the structural changes induced by Wallerian degeneration. Thus a complete description of Wallerian degeneration in immature white matter will not be attempted The features of Wallerian degeneration in adult central nervous tissue have been described by many workers (Luse and McCaman 1957 Luse 1960 Bignami and Ralston 1968 1969 Kruger and Maxwell 1969 Vaughn *et al* 1970 Vaughn and Pease 1970)

Degenerating fibres were seen within a well delimited region in the most dorsomedial part of the gracile fasciculus In the field of degeneration a few normal fibres persisted Outside the degenerating field the white matter appeared completely normal and was used as control. In the degenerating field the picture was dominated by great amounts of flattened and folded myelin sheaths with a normal lamellar pattern Many myelin-like globules of varying size were also present (Figs. 36, 37 and 38) showing either the normal myelin structure or a lamellar pattern similar to that in myelinoid bodies. Some myelin sheaths contained what appeared as dense degenerating axoplasm, while others were distended surrounding an empty space Many oligodendrocytes were partly covered by myelin in analogy with the redundant myelin sheaths of normal animals (Figs. 36 38 and 43) except that the glial myelin covering was not continuous with the myelin sheath of a normal fibre. Myelin-covered glial cells resembling the dense degenerating oligodendrocytes which occurred normally were noted. Degenerating glial cells which were not included within a myelin sheath also occurred (Figs. 39 40 and 42) Some myelin-covered glial cells were similar to microglia (Fig 44) and in many instances classification was impossible. Inclusion-containing microglial cells were frequent. The inclusions showed the same characteristics as in the normal



Text fig. 1 Length of the spinal cord (from the level of foramen magnum to the lower border of the seventh lumbar segment) plotted against age in 44 kittens and adult cats.

material (Figs. 41 42 and 45) In the periphery of the degenerating field mitotic figures were common.

In the area of the gracile fasciculus not affected by the operation many small myelinated fibres were seen. Here a few scattered myelinoid bodies and occasional degenerating glial cells were noted. Inclusion-containing microglial cells occurred but only incidentally No "myelinated" oligodendrocytes were observed in this region and redundant myelin sheaths were rare

Growth in length of the spinal cord

The results of the measurements are presented in Text fig 1 As illustrated by the diagram the length from the level of foramen magnum to the lower border of the seventh lumbar segment increased from around 90 mm at birth to approximately 310 mm (?70—310) in the adult stage Thus the postnatal longitudinal growth amounts to 3 4 times (cf Mellstrom and Skoglund 1969)

Discussion

Myelin sheath irregularities and so called myelin droplets have been observed by other workers both in immature white matter (Hess and Young 1952, Fleischhauer and Wartenberg 1967) and in cultivated central nervous tissue (Hild 1957 Bornstein and Murray 1958 Storts and Koestner 1969)

"Myelinated oligodendrocytes have been noted in some regions of the adult central nervous system (Leonhardt 1969 Rosenbluth 1966) and in tissue cultures (Field *et al.* 1968) Oligodendrocytes partly surrounded by myelin were seen during Wallerian degeneration (cf Howell and Kidd 1969 Bignami and Ralston 1969 Vaughn and Pease 1970) and Hirano *et al* (1968) observed similar formations after cyanide intoxication.

In specimens from normal kittens less than three weeks old, oligodendrocytes with an appearance strongly indicative of degeneration were encountered. The degenerative state of these cells is supported by the striking structural similarities to degenerating cells from various tissues and species as depicted by Scharrer (1966) Maruyama *et al* (1968) Campbell (1968) and Webster and Gross (1970) In a light microscopic work Spatz (1918) also noted degenerating glial cells in the myelinating spinal cord white matter and in the electron microscope Mori and Leblond (1970) observed degenerating oligodendroglia in the central nervous system of young rats.

The incidence of glial cells with a dense or disintegrated cytoplasm and a pyknotic nucleus resembling degenerating oligodendrocytes was increased

during Wallerian degeneration as compared to normal tissue. Frequently such degenerating glial cells were partly surrounded by myelin. Daniel and Strich (1969) also noted necrotic cells surrounded by myelin during Wallerian degeneration. Oligodendroglial cells degenerate in experimental allergic encephalomyelitis (Howell and Kidd 1969 Raine and Bornstein 1970 a, b) and are reduced in number or absent in the lesions of various demyelinating conditions (Courville 1957 Friede 1961 Bunge *et al.* 1961 Bornstein 1963 Périer and Grégoire 1965 Bunge and Glass 1965 Jellinger and Minauf 1969)

In peripheral nerves the reaction to Wallerian degeneration and demyelination seems to be different in that the Schwann cells proliferate and rapidly phagocytize myelin fragments and axonal remnants (Abercrombie and Johnson 1946 Kline *et al* 1958 Nathaniel and Pease 1963 a, b Prineas *et al* 1969 Seiler and Schröder 1970) whereas in central fibre tracts it is the microglial phagocytes that proliferate and ingest degenerating material (Luse 1960 Blinzinger and Hager 1964 Bunge and Glass 1965 Friede 1968 Bignami and Ralston 1969 Howell and Kidd 1969)

During the first three postnatal weeks many microglial cells were observed in the region of white matter used for this study These cells either occupied a perivascular position or were located within the nervous tissue and often contained cytoplasmic bodies similar to lysosomes (cf Mori and Leblond 1969) Other varieties of microglia, contained large polymorphous inclusions, whereas the lysosome-like cytoplasmic bodies were very few or absent. Accordingly Del Rio-Hortega (1932) described an invasion of the central nervous system at about the time of birth by ameboid microglial cells originating from the meninges and bloodvessels (cf Sakla 1965) In the superficial white matter of the newborn rabbit and kitten he observed a considerable number of microglia which as they entered the white matter began a phagocytic function and were rapidly transformed into globose fat-granule cells. When myelination was more complete the microglial cells gradually disappeared and were therefore less frequent in the adult stage. In the mature animals no globose fat granule cells were seen (Del Rio-Hortega 1932)

Several of the early light microscopists likewise pointed out the presence of so called fat globule cells during myelination in various species, including man. This phenomenon was considered by some of these authors to represent a pathological process which was denoted *encephalitis interstitialis neonatorum*" (see Virchow 1867 Del Rio-Hortega 1932) Others believed that the fat globules were myelin precursors being gradually transformed into myelin sheaths. A similar view was recently held by Barlow (1968) who, studying the immature sheep, stated that lipid droplets were continuously incorporated into the developing myelin (cf. Adams and Davison 1959 Mickel and Gilles

1970) However the generally accepted concept of myelin formation by spiral wrapping (Geren 1954 1964) has invalidated the older views (cf Berthold and Skoglund 1968 b)

A further argument against the thought of lipid droplets in the immature white matter as myelin precursors is that most of the myelin in one internode is formed late postnatally The lipid droplets however are largely limited to the period of early myelination (Adams and Davison 1959 Barlow 1968) The large fibres in the area used for the present study measure around 2.5 μ at birth, 5 μ at three weeks, 12.5 μ at three months and about 15 μ in the adult (Hildebrand and Skoglund 1971) A simple calculation will show that the amount of myelin in one future large internode increases 6 times during the first three weeks. By three months it has increased 78 times and in the adult 136 times as compared to the newborn stage This is an approximation and subject to several errors being based on the internodal distances in the rabbit (Hess and Young 1932) and on the assumption that the myelin sheath can be regarded as a tube. Nevertheless the tendency for most of the myelin in one internode to form late postnatally is clear Thus it seems unlikely that the lipid content of the early postnatal fat-granule cells represents myelin precursors. Instead the lipid inclusions might be derived from phagocytized material. This interpretation is favoured by the formation of fat granule cells in for example Wallerian degeneration (see Daniel and Strich 1969)

The lamellated microglial inclusions denoted here as type I might represent ingested myelin. This suggestion is supported by the high incidence of lamellated glial inclusions in Wallerian degeneration (Luse 1960, Bignami and Ralston 1969 Howell and Kidd 1969 Vaughn *et al.* 1970) It is well known that injury to central nervous tissue by trauma or disease is accompanied by microglial proliferation and an increase in microglial lysosomes (Field 1961 Blinzinger and Hager 1964 Cammermeyer 1965 a, b Hager 1968) Lipid laden phagocytes have been observed in a wide variety of central nervous disorders such as experimental allergic encephalomyelitis (Luse 1960 Lampert 1967) experimental spongy degeneration (Lampert and Schochet 1968) metachromatic leucodystrophy (Resibois 1968) Tay-Sachs disease (Terry and Weiss 1963 Wallace *et al* 1963) diffuse sclerosis (Nelson *et al.* 1962) progressive multifocal leucoencephalopathy (Headington and Umiker 1962) and multiple sclerosis (Friede 1961 Suzuki *et al.* 1969)

Since microglial phagocytes ingest necrotic cells (Blinzinger and Hager 1964) it seems reasonable to assume that the other varieties of microglial inclusions observed in the present report (types II, III and IV) represent phagocytized necrotic oligodendroglial cells in various stages of degradation.

On the basis of the results presented in this study and the discussion above

it is tentatively inferred that during the first three postnatal weeks a spontaneous myelin sheath disintegration takes place in the investigated area of the feline spinal cord white matter At an axon diameter around 1 μ the highly irregular myelin sheaths of some short internodes leave their axons. The two neighbouring myelin sheaths slowly elongate to cover the demyelinated segment and meet at a new node of Ranvier (cf Berthold and Skoglund 1968 b) The highly distorted and fragmenting discarded myelin sheath is phagocytized by microglia. Simultaneously some oligodendrocytes degenerate and eventually become ingested by microglial phagocytes. It seems likely that the degenerating oligodendrocytes and the disintegrating myelin sheaths to some extent were connected in myelin-glial units before the demyelination, this connection becoming broken at the onset of or during the course of the degenerative process. Within the microglial cells the final degradation of the ingested myelin and the necrotic cells takes place. The finding of several lysosome-like cytoplasmic bodies in the interstitial and perivascular microglia without inclusions whereas the inclusion-containing microglial cells have few or no discrete lysosome-like cytoplasmic bodies indicates that these might be involved in the degradation of the inclusion material (cf Cohn *et al.* 1963 Blinzinger and Hager 1964 Sutton and Weis 1966 Hager 1968)

The results upon which the hypothesis presented above was founded support the findings of Berthold and Skoglund (1968 a, b) in the peripheral nervous system. Their work formed part of the basis for the present study In the lumbar ventral roots from kittens less than three weeks old they observed a myelin sheath disintegration which prevailed in the paranodal regions. Furthermore some internodes, the myelin sheaths of which were highly distorted by multiple outgrowths and lamellated fragments, were very short. In other instances a Schwann cell loaded with lamellated fragments was juxtaposed to a naked axon segment. This was interpreted to be indicative of a degenerative process by which some internodes were removed from the affected fibres. The original interpretation by Berthold and Skoglund (1968 b) has gained much support from further studies of immature peripheral nerve fibres in several species (Berthold, to be published) Obviously the features of the immature white matter described in the present report to a certain extent correspond to the findings of Berthold and Skoglund cited above.

Since the pattern described in the present report was encountered in all kittens less than three weeks old although they came from different litters of varying geographical origin, it appears highly unlikely that the degenerative features would be caused by disease or intoxication (cf Berthold and Skoglund 1968 b) the more so since the animals used always appeared healthy It might be argued that the necrotic cells and the various myelin irregulari

ties are artefacts caused by fixation or other preparative procedures. Certainly myelin irregularities as well as dense shrunken cells can be mere products of the technique used. However short distorted internodes and degenerating oligodendroglial cells were never observed in older kittens. Furthermore the short internodes were limited to a certain fibre size It is also difficult to explain the presence during the first postnatal fortnight of numerou microglial cells, containing either lysosome like cytoplasmic bodies or large in clusions in terms of preparative artefacts.

It is known that programmed cell death plays an important role during early morphogenesis (see Källén 1965 Lockshin and Williams 1965 Webster and Gross 1970) Possibly the oligodendroglial necrosis and myelin sheath disintegration observed are manifestations of such a process. In that case the primary change resides in the myelin-bearing cell whereas the myelin sheath disintegration and subsequent demyelination represent secondary phenomena

Myelin sheath disintegration in short internodes appeared at an axonal diameter around 1 μ in central fibres. In immature peripheral fibres the corresponding process seems to occur at a larger axon diameter (Berthold and Skoglund 1968 b) It is well established that central axons myelinate at a diameter of 0.5 μ or somewhat less (Gaze and Peters 1961 Fleischhauer and Wartenberg 1967 Matthews 1968) whereas peripheral fibres acquire myelin sheaths at a size of 1 μ (Peters and Muir 1959) Provided that the initial internodal length is the same and that the subsequent elongation of the inter nodes with increasing fibre size follows the same time course in peripheral and central *fibres (cf Vizoso and Young 1948 Hess and Young 1952) the differ* ent sizes at which myelin sheath disintegration occurs would follow from the differing diameters at the initial myelination. At the newborn stage the largest fibres which measure 2—3 μ (Hildebrand and Skoglund 1971) probably have passed through the process of myelin sheath disintegration. The presence of degenerating oligodendrocytes and microglial inclusions in the newborn stage also indicates that the process has been going on for some time

In the central nervous system of kittens and newborn rabbits the minimal internodal length is around 200 μ according to light microscopic studies by *Nakai (1954)* and Hess and Young (1952) The latter authors stated that the correlation between internodal length and fibre diameter was less obvious in the newborn rabbit than in older animals. Since the smallest fibres mea sured by them had a diameter around 2 μ a minimal nodal spacing of 200 μ is not necessarily valid for smaller fibres (cf Haug 1967) Thus internodes varying in length between 4 and 70 μ and long unmyelinated interruptions of otherwise myelinated fibres were noted by Storts and Koestner (1969) in tissue cultures of canine cerebellum.

The majority of the myelinated fibres studied in the lateral funiculus of the newborn range around 1 μ. Some fibres reach 2 μ and very few exceed that size (Hildebrand and Skoglund 1971). Since the largest fibres in the same region of adult animals measure 14—17 μ (Hildebrand and Skoglund 1971) it is reasonable to assume that the 2 μ fibres of the newborn will reach an adult size of at least 12 μ. Hess and Young (1952) found that in the new born rabbit the internodal distances of 2 μ spinal cord fibres were approximately 200—300 μ. Adult rabbit fibres with a diameter of 12 μ showed a mean internodal length around 1300 μ. If these figures are applied to the cat it appears that the postnatal internodal longitudinal growth of the fibres under consideration amounts to at least 4.3—6.5 times. This exceeds the growth in length of the spinal cord. In order to be proportionate to the spinal cord growth of 3.4 times found in this report the internodes along a fibre with an adult nodal spacing of 1300 μ should have a mean length of nearly 400 at birth. The figures above are hypothetical since the internodal distances relied upon are derived from the rabbit.

As discussed above it seems that a number of internodes are removed from developing future large fibres at an axon diameter around 1 μ which corresponds to a fibre size of approximately 1.2 μ (Hildebrand 1971 a). A disappearance of some internodes would result in unmyelinated segments along otherwise myelinated fibres allowing the proportional elongation of the remaining internodes to exceed that of the spinal cord. In 2 μ fibres the covering of demyelinated segments by growth of remaining internodes is probably well under way but not necessarily completed. Such a process would explain the discrepancy between internodal elongation and spinal cord growth in length and perhaps also the less strict correlation between fibre size and internodal length in immature as compared to adult spinal cord fibres (Hess and Young 1952). Berthold and Skoglund (1968 b) found a similar pattern in developing feline peripheral nerves.

According to electrophysiological investigations myelinated fibres in the immature central nervous system conduct impulses at a very low velocity and have a low capacity to follow rapid stimulation (Purpura *et al.* 1964, Scherrer 1967, Conway *et al.* 1969). As discussed elsewhere (Hildebrand 1971 a, Hildebrand and Skoglund 1971) developing future large central fibres seem to become close to physiologically mature at a size around 4—5 μ in analogy with developing peripheral nerves (see Skoglund and Romero 1965). At this fibre size the nodes of Ranvier in future large fibres have attained a nearly adult structure (Hildebrand 1971 a). It was thought that the rapid change in conduction velocity and the increasing ability to maintain sustained activity at a fibre size around 4—5 μ (Purpura *et al.* 1964, Huttenlocher 1970) would

somehow be related to the simultaneously occurring nodal maturation (Hildebrand 1971 a) In developing peripheral nerves the function and the nodal-paranodal morphology are intimately correlated to each other and to the fibre size (Skoglund and Romero 1965 Berthold and Skoglund 1965 1967 1968 a, b, Berthold 1968 b)

Possibly the slow conduction velocity and the limited capacity to follow rapid stimulation of immature myelinated central fibres is not solely related to the undifferentiated structure of the nodes of Ranvier at that stage. The early postnatal myelin sheath disintegration and subsequent demyelination suggested by the present results, leaving long unmyelinated segments between the remaining internodes, introduces another factor which might be of importance with respect to function As demonstrated by McDonald and Sears (1969 1970) and Harrison *et al* (1970) a localized experimental demyelination in the feline spinal cord white matter results in conduction block or a greatly reduced conduction velocity of the affected fibres The ability to follow rapid stimulation is also reduced.

The final effect of a disappearance of a number of internodes in a myelinated fibre should be a proportionate reduction of the number of nodes of Ranvier and a greater nodal spacing due to elongation of remaining internodes. As discussed by Berthold and Skoglund (1968 b) such a process would eventually increase the conduction velocity of the affected fibres (Rushton 1951)

Summary

The superficial white matter in the lateral funiculus of the first cervical spinal cord segment was studied in kittens fixed by glutaraldehyde perfusion During the first postnatal fortnight myelin sheath irregularities, myelin fragments and myelinoid bodies were common. Some internodes were very short and showed a highly distorted myelin sheath Long unmyelinated segments of otherwise myelinated fibres also occurred and sometimes were surrounded by myelin fragments. A number of necrotic cells interpreted as degenerating oligodendrocytes were seen. Several microglial cells were present containing either lysosome like bodies or large polymorphous inclusions which apparently represented ingested myelin and phagocytized necrotic cells. Similar features were common during Wallerian degeneration. At the end of the third postnatal week these features were less frequent and laregly absent in older animals. Myelinoid bodies were however commonly seen also in older kittens and in adult cats.

The findings were interpreted as signs of an early postnatal breakdown of myelin-glial units with myelin sheath disintegration and segmental demyelina

tion in future large myelinated fibres. This would explain the discrepancy between spinal cord growth in length and internodal elongation in future large fibres. The results were discussed in the light of similar findings in immature peripheral nerves and neuropathological and physiological data.

Acknowledgements

This investigation was supported by grants from Karolinska Institutet (Reservationsanslaget) and from the Swedish Medical Research Council (projects no. B70-14X-136-06 and B71 14X 136-07A). I am much indebted to Professor Sten Skoglund for his encouragement and constructive criticism and to Mrs. Ingegerd Lindqvist for excellent technical assistance.

References

ABERCROMBIE, M. and JOHNSON, M. L. Quantitative histology of Wallerian degeneration. I. Nuclear population in rabbit sciatic nerve. *J Anat* *80* 37—50 (1946)

ADAMS, C. W. M. and DAVISON, A. N. The occurrence of esterified cholesterol in the developing nervous system. *J Neurochem* *4* 282—289 (1959)

BARLOW, R. M. The foetal sheep. Morphogenesis of the nervous system and histochemical aspects of myelination. *J Comp Neurol.* *135* 249—262 (1969)

BERTHOLD, C. H. A study on the fixation of large mature feline myelinated central lumbar spinal-root fibres. *Acta Soc. Med. Upsal.* *73* Suppl. 9 1—36 (1968 a)

BERTHOLD, C. H. Ultrastructure of postnatally developing peripheral nodes of Ranvier. *Acta Soc Med Upsal.* *73* 145—168 (1968 b)

BERTHOLD, C. H. and SKOGLUND, S. Ultrastructure and histochemistry of the developing node of Ranvier in the hindlimb nerves of the cat. *Acta Soc Med Upsal.* *70* 287—293 (1965)

BERTHOLD, C. H. and SKOGLUND, S. Histochemical and ultrastructural demonstration of mitochondria in the paranodal region of developing feline spinal roots and nerves. *Acta Soc Med. Upsal.* *7*? 37—70 (1967)

BERTHOLD, C. H and SKOGLUND, S. Postnatal development of feline paranodal myelin-sheath segments. I. Light microscopy. *Acta Soc Med. Upsal.* *73* 113—126 (1968)

BERTHOLD, C. H. and SKOGLUND, S. Postnatal development of feline paranodal myelin-sheath segments. II. Electron microscopy. *Acta Soc Med Upsal.* *73* 127—144 (1968 b)

BIGNAMI, A. and RALSTON, H. J. Myelination of fibrillary astroglial processes in long term Wallerian degeneration. The possible relationship to status marmoratus. *Brain Res* *11* 710—713 (1968)

BIGNAMI, A. and RALSTON, H. J. The cellular reaction to Wallerian degeneration in the central nervous system of the cat. *Brain Res.* *13* 444—461 (1969)

BLINZINGER, K. and HAGER, H. Elektronenmikroskopische Untersuchungen zur Feinstruktur ruhender und progressiver Mikrogliazellen im Zentralnervensystem des Goldhamsters. pp. 99—112 In *Progress in Brain Research* *6* (W. Bargmann and J. P. Schadé eds.) Elsevier, Amsterdam (1964)

Bodian D. Spontaneous degeneration in the spinal cord of monkey fetuses. *Bull. Hopk. Hosp* 119 212—234 (1966)

Bornstein M. B. A tissue-culture approach to demyelinative disorders. pp 197—214 in *Symposium on Organ Culture* Nat. Cancer Inst. Monograph 11 (C. J. Dawe ed.) Washington (1963)

Bornstein M. B. and Murray M. R. Serial observations on patterns of growth, myelin formation, maintenance and degeneration in cultures of newborn rat and kitten cerebellum. *J. Biophys. Biochem. Cytol.* 4 449—504 (1958)

Brody I. The keratinization of epidermal cells of normal guinea pig skin as revealed by electron microscopy *J. Ultrastruct. Res.* 2 482—511 (1959)

Bunge, M. B. Bunge, R. P. and Ris H. Ultrastructural study of remyelination in an experimental lesion in adult cat spinal cord *J. Biophys. Biochem. Cytol.* 10 67—94 (1961)

Bunge, M. B. Bunge, R. P. and Pappas, G. D. Electron microscopic demonstration of connections between glia and myelin sheaths in the developing mammalian central nervous system. *J. Cell Biol.* 1 448—453 (1962)

Bunge, R. P. and Glass, P. M. Some observations on myelin-glial relationships and on the etiology of the cerebrospinal fluid exchange lesion. *Ann. N. Y. Acad. Sci.* 12 15—28 (1965)

Caley D. W. and Maxwell, D. S. An electron microscopic study of the neuroglia during postnatal development of the rat cerebrum. *J. Comp. Neurol.* 133 45—70 (1968)

Cammermeyer, J. Juxtavascular karyokinesis and microglia cell proliferation during retrograde reaction in the mouse facial nucleus. *Ergebn. Anat. Entwickl. gesch.* 38 1—22 (1965 a)

Cammermeyer, J. Histiocytes, juxtavascular mitotic cells and microglia cells during retrograde changes in the facial nucleus of rabbits of varying age. *Ergebn. Anat. Entwickl. gesch.* 38 195—229 (1965 b)

Campbell, F. R. Nuclear elimination from the normoblast of fetal guinea pig liver as studied with electron microscopy and serial sectioning technique *Anat. Rec.* 160 539—554 (1968)

Cohn, Z. A., Hirsch, J. G. and Wiener, E. Lysosomes and endocytosis. The cytoplasmic granules of phagocytic cells and the degradation of bacteria. pp. 126—150 in *Ciba Foundation Symposium on Lysosomes* (A. V. S. de Reuck and M. P. Cameron eds.) Churchill, London (1963)

Conway C. J. Wright F. S. and Bradley W. E. Electrophysiological maturation of the pyramidal tract in the postnatal rabbit *Electroenceph. Clin. Neurophysiol.* 26 565—577 (1969)

Courville, C. B. The process of demyelination in the central nervous system. IV. Demyelination as delayed residual of CO-asphyxia. *J. Nerv. Ment. Dis.* 125 534—546 (1957)

Daniel, P. M. and Strich S. J. Histological observations on Wallerian degeneration in the spinal cord of the Baboon, Papio Papio. *Acta Neuropath.* (Berlin) 12 314—328 (1969)

Del Rio-Hortega, P. Microglia. pp. 483—529 in *Cytology and Cellular Pathology of the Nervous System* 2 (W. Penfield ed.) Hoeber New York (1932)

Ernst M. Über Untergang von Zellen während der normalen Entwicklung bei Wirbeltieren. *Z. Anat. Entwickl. gesch.* 79 228—262 (1926)

FIELD E. J The early lesion of experimental allergic encephalomyelitis. *Exp Neurol.* 4 233—240 (1961)

FIELD E. J HUGHES, D and RAINE, C. S Electron microscopic observations on the development of myelin in cultures of neonatal rat cerebellum. *J Neurol. Sci.* 8 49—60 (1968)

FLEISCHHAUER, K. and WARTENBERG, H. Elektronenmikroskopische Untersuchungen über das Wachstum der Nervenfasern und über das Auftreten von Markscheiden im Corpus callosum der Katze. *Z. Zellforsch.* 83 568—581 (1967)

FRIEDE, R. L. Enzyme histochemical studies in multiple sclerosis. *Arch. Neurol.* 5 433—443 (1961)

FRIEDE, R. L. Histochemische Befunde zur Gliafunktion. *Acta Neuropath* (Berlin) Suppl. IV 53—54 (1968)

FRIEDE, R. L. and SAMORAJSKI, T Axon caliber related to neurofilaments and microtubules in sciatic nerve fibres of rats and mice. *Anat Rec* 167 379—388 (1970)

GAZE, R. M. and PETERS, A. The development, structure and composition of the optic nerve of Xenopus Laevis. *Quart. J Exp Physiol.* 46 299—309 (1961)

GEREN B. B. The formation from the Schwann cell surface of myelin in the peripheral nerves of chick embryos. *Exp Cell. Res.* 7 558—562 (1954)

GEREN-UZMAN B. B. The spiral configuration of myelin lamellae. *J Ultrastruct Res* 11 208—212 (1964)

GLÜCKSMANN, A. Cell deaths in normal vertebrate ontogeny *Biol. Reviews* 26 59—86 (1951)

GONATAS, N K., LEVINE, S. and SHOULSON, R. Electron microscopic investigation of phagocytosis in an experimental leucoencephalopathy *Ann. N Y Acad Sci.* 122 6—14 (1965)

HAGER, H. Pathologie der Makro- und Mikroglia im elektronenmikroskopischen Bild. *Acta Neuropath.* (Berlin) Suppl. IV 86—97 (1968)

HAMBURGER, V Development of the nervous system *Ann N Y Acad. Sci.* 55 117—132 (1952)

HARRISON B. M., MCDONALD, W L, OCHOA, J and SEARS, T A. Electron microscopic observations on a focal experimental demyelinating lesion in the cat spinal cord. *J Neurol. Sci.* 10 409—413 (1970)

HASHIMOTO, P H. Electron microscopic study of gliosome formation in postnatal development of the spinal cord in the cat. *J Comp. Neurol.* 137 251—255 (1969)

HAUG, H. Die Länge der Internodien der Markfasern im Bereich der Sehrinde der erwachsenen Katze *Z Zellforsch.* 83 265—278 (1967)

HEADINGTON J T and UMIKER, W O Progressive multifocal leucoencephalopathy A case report. *Neurology* (Minneap.) 12 434—439 (1962)

HESS, A. and YOUNG, J Z. The nodes of Ranvier *Proc Roy Soc B.* 140 301—320 (195)

HILD W Myelogenesis in cultures of mammalian central nervous tissue. *Z Zellforsch.* 46 71—95 (1957)

HILDEBRAND C. Ultrastructural and light-microscopic studies of the developing feline spinal cord white matter I. The nodes of Ranvier *Acta Physiol. Scand* Suppl. 364 81—108 (1971)

HILDEBRAND, C. Ultrastructural and light-microscopic studies of the nodal region in large myelinated fibres of the adult feline spinal cord white matter *Acta Physiol. Scand* Suppl 364 43—80 (1971 b)

HILDEBRAND, C. and SKOGLUND S. Calibre spectra of some fibre tracts in the feline central nervous system during postnatal development. Acta Physiol Scand. Suppl. 364 5—42 (1971)
HIRANO, A., LEVINE, S. and ZIMMERMAN H. M. Remyelination in the central nervous system after cyanide intoxication. J Neuropath Exp Neurol 27 234—245 (1968)
HOWELL, J. I. and KIDD M. An electron microscopical comparison of primary and secondary demyelination in the rat central nervous system. Virchows Arch. Abt B Z Zpath. 187—207 (1969)
HUGHES, A. F. LOAR M. and TURNER T. Degeneration of nerve fibres within the embryonic spinal cord. Nat 221 579—581 (1969)
HUTTENLOCHER P. Myelination and the development of function in immature pyramidal tract. Exp Neurol. 29 405—415 (1970)
JELLINGER, K. and MOSAUR M. Eigenartige Entmarkungskrankheit im Erwachsenalter. Arch. Psychiat Nervenk. 212 371—381 (1969)
KARLSSO K. Comparison of the myelin period of peripheral and central origin by electron microscopy. J Ultrast. Res 15 451—468 (1966)
KLINE D. MAGEE, W. L., PRITCHARD, E. T. and ROSSITER, R. J. Chemical studies of peripheral nerve during Wallerian degeneration — VII. Labelling of phospholipid and cholesterol from acetate. J Neurochem 3 52—58 (1958)
KRUGER, L. and MAXWELL, D. Electron microscopy of oligodendrocytes in normal rat cerebrum. Amer J Anat 118 411—437 (1966)
KRUGER, L. and MAXWELL, D. Wallerian degeneration in the optic nerve of a reptile. An electron-microscopic study. Am J Anat 125 247—270 (1969)
KÄLLÉN B. Degeneration and regeneration in the vertebrate central nervous system during embryogenesis. pp 77—96 in Progress in Brain Research 14 (M. Singer and J. P. Schadé eds.) Elsevier Amsterdam (1965)
LAMPERT P. W. Electron microscopic studies on ordinary and hyperacute experimental allergic encephalomyelitis. Acta Neuropath. (Berl.) 9 99—126 (1967)
LAMPERT P. W. and SCHOCHET S. S. Electron microscopical observations on experimental spongy degeneration of the cerebellar white matter. J Neuropath Exp Neurol. 27 210—220 (1968)
LEONHARDT H. Myelinisierte Oligodendrocyten in der Wand der Eminentia mediana des Kaninchens. Z Zellforsch 103 420—428 (1970)
LEVI-MONTALCINI R. Events in the developing nervous system. pp 1—30 in Progress in Brain Research 4 (D. P. Purpura and J. P. Schadé eds.) Elsevier Amsterdam (1964)
LOCKSHIN, R. A. and WILLIAMS, C. M. Programmed cell death — I. Cytology of degeneration in the intersegmental muscles of the pernyi silkmoth. J Ins Physiol 11 123—133 (1965)
LUSE S. A. Experimental demyelination: an electron microscopic study. Anat Rec 136 236—237 (1960)
LUSE, S. A. and MCCAMAN, R. E. Electron microscopy and biochemistry of Wallerian degeneration in the optic and tibial nerves. Amer J Path 33 586 (1957)
MARUYAMA, S. CIRIO, M. and D'AGOSTINO, A. N. Selective necrosis in the fetal rat central nervous system produced by 5-fluoro-2'-deoxyuridine. A morphologic study. J Neuropath. Exp Neurol. 27 96—107 (1968)
MATTHEWS M. A. An electron microscopic study of the relationship between axon diameter and the initiation of myelin production in the peripheral nervous system.

An t R *161* 337—35 (1968)

MAUNSBACH, A. B Observations on the ultrastructure and acid phosphatase activity of th cytoplasmic bodies in rat kidney proximal tubule cells *J Ult astruct Res. 16* 197—238 (1966)

MAXWELL, D and KRUGER, L. The reactive oligodendrocyte. An electron microscopic study of cerebral cortex following lpha-particle irradiation. *Amer J Anat 118* 437—461 (1966)

McDONALD W L and SEARS, T A. Effect of demyelination on conduction in the central nervous system. *Nat e 221* 182—183 (1969)

McDONALD W I and SEARS, T A. Effect of a dem linating lesion on cond ction in the central nervous sy tem studied in single nerve fibres. *J Physiol.* (London) *207* 53—54 (1970)

MELLSTRÖM A and SKOOLUND S. Quantitative morphological changes in some spinal cord segments during postnatal development. A study in the cat. *A t Physol. Sc d* Suppl. 331 1—84 (1969)

MICKEL, H. S. and GILLES F H. Changes in glial cells during human telencephalic myelinogenesis. *B n 93* 337—346 (1970)

MORI, S. and Leblond C. P Identification of microglia in light and electron microscopy *J Comp Neur L 135* 57—80 (1969)

MORI, S and LEBLOND P Electron microscopic identification of three classes of oligodendrocytes and preliminary tudy of their proliferative activity in the corpus callosum of young rats. *J Comp Ner ol. 139* 1—30 (1970)

NAKAI, J Th osmic acid injection method for demonstrating nodes in the central nervous system. *Anat R c. 119* 267—274 (1954)

NATHANIEL, J H. and PEASE, D C Degenerative changes in rat dorsal roots during Wallerian degeneration *J Ultrastru t Res. 9* 511—532 (1963)

N THANIEL, J H and PEASE D C. Regenerative changes in rat dorsal roots following Wallerian degeneration *J Ult astruct R 9* 533—549 (1963 b)

NELSON E OSTERBERG, K BLAW M STORY J and KOZAK, P Electron microscopic and histochemical tudies in diffuse sclerosis (sudanophilic type) *Neurology* (Minneap) *12* 896—909 (1962)

PENFIELD W and OXON B Oligodendroglia and its relation to classical neuroglia. *Brain 47* 430—452 (1924)

PÉRIER, O and GRÉGOIRE, A. Electron microscopic features of multiple sclerosis lesions. *B ai 88* 937—85 (1965)

PETERS, A Further observations on the structure f myelin sheaths in the central nervous system. *J C ll Biol. 20* 281—296 (1964)

PETERS A and MUIR A. R The relationship between axons and Schwann cells during development of peripheral nerves in the rat *Q art J E p Physiol 44* 117—130 (1959)

PRINEAS, J RAINE, C. S and WISNIEWSKI, H. An ultrastructural study of experimental demyelination and remyelination. III Chronic experimental allergic encephalomyelitis in the central nervou system. *Lab I vest. 21* 47 —483 (1969)

PURPURA, D P SHOFER, R. J HOUSEPIAN E M. and NOBACK C R Comparativ ontogenesis of structure function relations in cerebral and cerebellar cortex pp 187—221 in *P gress in Brain Researc h 4* (D P Purpura and V P Schadé eds) Elsevier Amsterdam (1964)

RAINE, C. S. and BORNSTEIN, M. B Experimental allergic encephalomyelitis an ultra-

structural study of experimental demyelination in vitro. *J. Neuropath. Exp. Neurol.* 29 177—191 (1970 a)

RAINE, C. S. and BORNSTEIN, M. B. Experimental allergic encephalomyelitis: a light and electron microscope study of remyelination and "sclerosis" in vitro. *J. Neuropath. Exp. Neurol.* 29 552—574 (1970 b)

RESIBOIS, A. Electron microscopic study of metachromatic leucodystrophy. III. Lysosomal nature of the inclusions. *Acta Neuropath.* (Berlin) *13* 149—156 (1969)

REYNOLDS, E. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* *17* 208—212 (1963)

ROBERTSON, J. Redundant myelin sheaths and other ultrastructural features of the toad cerebellum. *J. Cell Biol.* *28* 73—93 (1966)

RUSHTON, W. A. H. A theory of the effects of fibre size in medullated nerve. *J. Physiol.* (London) *115* 101—122 (1951)

RUSSELL, G. V. The compound granular corpuscle or gitter cell: a review, together with notes on the origin of this phagocyte. *Tex. Rep. Biol. Med.* *20* 338—351 (1962)

RYTER, A. and KELLENBERGER, E. L'inclusion au polyester pour l'ultramicrotomie. *J. Ultrastruct. Res.* 2 200—214 (1958)

SARLA, F. B. Postnatal growth of neuroglia cells and blood vessels of the cervical spinal cord of the albino mouse. *J. Comp. Neurol.* *124* 189—202 (1965)

SCHARRER, B. Ultrastructural study of the regressing prothoracic glands of blattarian insects. *Z. Zellforsch.* *69* 1—1 (1966)

SCHERRER, J. Electrophysiological aspects of cortical development. pp. 480—489. In *Progress in Brain Research* *22* (P. A. Asratyan ed.) Elsevier, Amsterdam (1967)

SEILER, N. and SCHRÖDER, J. M. Beziehungen zwischen Polyaminen und Nucleinsäuren. II. Biochemische und feinstrukturelle Untersuchungen am peripheren Nerven während der Wallerschen Degeneration. *Brain Res.* 2 81—103 (1970)

SKOGLUND, S. and ROMERO, C. Postnatal growth of spinal nerves and roots. *Acta Physiol. Scand.* *66* Suppl. 260 1—50 (1965)

S[illegible]TZ, H. Beiträge zur normalen Histologie des Rückenmarks des neugeborenen Kaninchens mit Berücksichtigung der Veränderungen während der extrauterinen Entwicklung. *Histol. Histopath. Arb.* 6 478—604 (1918)

STOFFEL, R. W. and KOLSTNER, A. Development and characterization of myelin in tissue culture of canine cerebellum. *Z. Zellforsch.* *95* 9—18 (1969)

SUTTON, J. S. and WEISS, L. Transformation of monocytes in tissue culture into macrophages, epithelioid cells and multinucleated giant cells. *J. Cell Biol.* *28* 303—332 (1966)

SUZUKI, K., ANDREWS, J. M., WALTZ, J. M. and TERRY, R. D. Ultrastructural studies of multiple sclerosis. *Lab. Invest.* *20* 444—454 (1969)

TERRY, R. D. and WEISS, M. Studies in Tay-Sachs disease. II. Ultrastructure of the cerebrum. *J. Neuropath. Exp. Neurol.* *22* 18—55 (1963)

VAUGHN, J. E. An electron microscopic analysis of gliogenesis in rat optic nerve. *Z. Zellforsch.* *94* 293—324 (1969)

VAUGHN, J. E. and PETERS, A. Electron microscopy of the early postnatal development of fibrous astrocytes. *Amer. J. Anat.* *121* 131—152 (1967)

VAUGHN, J. E., HINDS, P. L. and SKOFF, R. P. Electron microscopic studies of Wallerian degeneration in rat optic nerves. I. The multipotential glia. *J. Comp. Neurol.* *140* 175—206 (1970)

VAUGHN, J. E. and PEASE, D. C. Electron microscopic studies of Wallerian degeneration

in rat optic nerves. II Astrocytes, oligodendrocytes and adventitial cells. *J Comp. Neurol. 140* 207—227 (1970)

VIRCHOW R. Zur pathologischen Anatomie des Gehirns. I. Congenitale Encephalitis und Myelitis. *Virchows Arch. Path. Anat. 38* 129—138 (1867)

VIZOSO, A. D and YOUNG, J Z. Internode length and fibre diameter in developing and regenerating nerves. *J Anat* (London) *82* 110—134 (1948)

WALLACE B. J VOLK, B. W and LAZARUS, S. S Glial cell enzyme alterations in infantile amaurotic family idiocy (Tay-Sachs disease) *J Neurochem. 10* 439—446 (1963)

WEBSTER, D A. and GROSS, J Studies on possible mechanisms of programmed cell death in the chick embryo. *Developmental Biol. 2* 157—184 (1970)

WECHSLER, W Elektronenmikroskopischer Beitrag zur Nervenzelldifferenzierung und Histogenese der grauen Substanz des Rückenmarks von Hühnerembryonen. *Z. Zellforsch. 74* 401—422 (1966 a)

WECHSLER, W Elektronenmikroskopischer Beitrag zur Histogenese der weissen Substanz des Rückenmarks von Hühnerembryonen. *Z Zellforsch 74* 232—251 (1966 b)

WECHSLER, W and MELLER, K. Electron microscopy of neuronal and glial differentiation in the developing brain of the chick. pp. 93—144 in *Progress in Brain Research 26* (C. G Bernhard and J P Schadé eds.) Elsevier Amsterdam (1967)

Legends to figures

Figs. 1—10 are light micrographs and Figs. 11—35 lectron micrographs of spinal cord white matter in normal kittens.

Fig. 1 a and b. Tw week old kitten. Longitudinal section through large f bre (A = axon) The section plane goes through the fibre in Fig 1 a. N t the oligodendrocyt (OL) close to the fibre Fig 1 b is a tangential section. Several compact myelin-lik bodies can be seen along the fibre. The oligodendrocyte (OL) has a broad process (arrow) which seems t be ttached to the fibre (1500 ×)

Fig. .. Two week old kitten. Longitudinal section through small fibre the left and right ends of which are myelinated. Between the arrow an unmyelinated segment urrounded by empty acuoles and dense fragments can be seen. (1500 ×)

Fig. 3. Newborn kitten. Transverse section. A large myelinated fibre (indicated by) is surrounded by several compact my lin-like bodies (arrows) (1500 ×)

Fig 4 Two week old kitten. Transverse section In the center of the picture large com pact my lin-like body (arrow) can be seen. (1500 ×)

Fig. 5 a and b. Newborn kitten. Longitudinal sections from the same region. Fig 5 a. The picture shows a glial cell surrounded by myelin (arrow) Fig 5 b. Th cell covered by myelin (arrow) is an oligodend ocyte. An opening in the my lin covering has appeared on the left side. Other sections in the same series showed that the glial myelin covering represented an extension of my lin sheath. I the upper right corner a ll containing a vacuolated inclusion can be noted (double arrow) (1500 ×)

Fig. 6 Newborn kitten. Longitudinal section. Arrow poi ts t glial cell with n irregular nucleus and dense inclusion. (1500 ×)

Fig. 7 Two week old kitten. Longitudinal section Arrow points to glial cell containing large nearly empty acuole. (1500 ×)

Fig. 8. Two week old kitten. Longitudinal section. A nucleated body (rrow) to the right of which a glial nucleus (N) can be seen (B = blood essel) (1500 ×)

Fig. 9 Newborn kitten. Longitudinal section. The picture shows ucleated body (arrow) Note the elongated oligodendrocyte (OL) t both ends of which a myelinated fibre falls within the plane of section. (1500 ×)

Fig. 10. Newborn kitten. Longitudinal section. Arrow points t another ariety of nucleated body (1500 ×)

Fig. 11 Thirteen day old kitten. Longitudinal section. Oligodendrocyte in early stage of degeneration. Note the paucity of organelles, the swollen mitochondria (m) and the vesiculated cytoplasm. The ucleus (N) is tangentially sectioned. (9120 ×)

Fig. 1 Thirteen day old kitten. Longitudinal section. Degenerating glial cell. The dense nucleus (N) is deeply invaginated t some places (arrows) and surrounded by thin rim of dense cytoplasm which was f lled with small ribosome-like granules. Note the mitochondrion (m) (11,800 ×)

Fig. 13 Two week old kitten Transverse section. Degenerating oligodendrocyte The spherical nucleus (N) is isolated by glial process (gp) Outside the nucleus two islands of dense vesiculated cytoplasm (larg arrow) are interconnected by thin membranous

strands (small arrows) N te the swollen mitochondrion (m) in the upper island of cytoplasm. (9150 ×)

Fig 14 Ten day old kitten. Longitudinal section. Degenerating oligodendrocyte. The elongated nucleus (N) contains large clumps of a dense material and is surrounded by a thin rim of dense vesiculated cytoplasm. Outside th cytoplasm glial process (gp) can be seen. Two small islands of the dense vesiculated cytoplasm (large arrows) have thin membranous interconnection. (12,600 ×)

Fig. 15 One week old kitten. Longitudinal section. Myelinated oligodendrocyte (OL) Not that the glial myelin covering is continuous with the sheath of myelinated fibre (large arrows) Between the myelin sheath and the axon (A) a cytoplasm with many small lamellated fragments is enclosed in the right part of the picture (small arrows)

Fig 16 One week old kitten. Longitudinal section. The myelinated fibre in the lower part of the picture (A = axon) shows a bizarre my lin sheath evagination which contains both an outgrowth from the axon ( ) and glial cytoplasm (g) (8900 ×)

Fig. 17 Newborn kitten. Longitudinal section. Part of a myelinated fibre is seen. The myelin sheath is separated from the axon (A) by an accumulation of glial cytoplasm (large arrows) which contains many small esicles. (16,800 ×)

Fig 18. Newborn kitten. Th myelin sheath of the cross-sectioned fibre (A = axon) is split. Between the outer layer and the layer which apposes the axon cytoplasm containing several granular lamellated and vesicular bodies (indicated by 1– 5) can be seen. (20,300 ×) Inset shows the area indicated by the rectangle t higher magnifica tion. The myelin sheath (M) shows normal lamellar pattern with major and minor dense lines. The included part of one of the lamellated bodies (B) has structure dif ferent from my lin. (79,300 ×)

Fig. 19 Newborn kitten Transverse section. Myelinated fibre (A = axon) with redundant myelin sheath. (9200 ×)

Fig 20 Newborn kitten. Transverse section. My linated fibre (A = axon) with highly irregular myelin sheath. Various cytoplasmic processes are interposed between the axon and the sheath. (17,900 ×)

Fig 21 a. Four day old kitten. Short internode (A = axon) The myelin sheath extends between large arrows. Not the loops of myelin given off from the sheath (small arrow) (5350 ×)

Fig 21 b. Same short internode as in Fig 21 a. Later in the series. The left end of the short internode is shown at higher magnification. Small arrows indicate terminal cytoplasmic pockets. Note their dense and somewhat vesiculated ppearance. A glial process (large arrow) enters in between the axon and the myelin sheath (here the axon is outsid the plane of sectioning) (15,370 ×)

Fig 22. Newborn kitten. Transverse section. Th picture shows an axon (A) surrounded by various processes and highly distended myelin sheath (M) outside which an aggregate of a dense granular and lamellar material is seen (arrow) (11 670 ×)

Fig. 23 One week ld kitten. Longitudinal section. Very short internode (between large arrows) The axon (A) is surrounded bl glial processes and an irregular myelin sheath with large outgrowths. Small arrows indicate terminal cytoplasmic pockets. (10,900 ×)

Fig. 21 a and b. One week old kitten. Longitudinal sections from the same series. Very short internode. Fig. 21 a. A von (A) is seen leaving highly expanded myelin sheath. The terminal cytoplasmic pockets (small arrows) have detached from the axon. A glial cytoplasm (g) and a large dense lamellated body (1) are enclosed within the myelin she th in addition to the von. Similar dense lamellated bodies (2 and 3) occur outside the sheath. An astrocyt is present in the vicinity (N indicates nucleus) (6450 x) Fig. 24 b. Later in the series. The plane of sectioning now goes through the von (A) in the other end of the short internode Arrow indicate where the myelin sheath terminates. (N = astrocytic nucleus) (8050 x)

Fig 25 Four day old kitten Longitudinal section Very short internode (arrows indicat terminal cytoplasmic pockets, A = von) The myelin sheath is highly irregular forming large loops (large arrows) Note the dense lamellated fragments (double small arrows) (9900 x)

Fig 26 One week old kitten. Longitudinal section. Large body composed of a material similar to my lm. (11,600 x)

Fig 27 One week old kitten. Transv rse section. The picture shows whorls of my lin (large arrows) to which esiculated fairly dense cytoplasm is associated (small arrows) A cytoplasmic process of similar ppearance connects the my lin loops with an axon (A) (double small arrows) (17,200 x)

Fig 28. Fou day old kitten. Longitudinal section Perivascular microglial cell (N = nucleus) Note the cytoplasmic bodies (types 1 and 3) (1.,400 x) Inset shows detail of microglial cytoplasmic body type 2. The inner leaflet of the lim ting membrane is thicker than the outer (arrows) A narrow light zone separates the matrix of the body from the membrane. (78,100 x)

Fig. 29 Ten day old kitten. Longitudinal section. An von (A) which is not covered by myelin but surrounded by myeli fragments within fai ly dense vesiculated cytoplasm (arrows) is seen. The cell in the upper part of the picture (ucleus indicated by N) is microglial cell. (11,900 x)

Fig. 30 Newborn kitten. Longitudinal section. Processes from microglial cell (arrows, nucleus indicated by N) can be seen on both sides of myeli fragment (M) Note the presence of other my lin fragments and an axon (A) which is not covered by myelin in the vicinity (9850 x)

Fig 31 Three week old kitten. Longitudinal section. Microglial cell (MG) containing large inclusion (Type I) of what appears to be myelin (MB) Note the "lipid droplets (L) (11 100 x) Inset shows the are indicated by the rectangle t higher magnification. (89.800 x)

Fig. 3. Ten day old kitten. Longitudinal section. Microglial cell with type II inclusion (arrow point to inclusion material) (19 100 x) Inset shows area indicated by the rectangle. The two bodies illustrated by the inset (m) resemble mitochondria. (63,000 x)

Fig. 33 Ten day old kitten. Longitudinal section. Microglial cell (nucleus indicated by N) with inclusion of type II The inclusion material indicated by small arrows is similar to the dense vesiculated cytoplasm in Figs. 13 and 14 Note how the microglial cytoplasm protrudes into the phagocytic vacuole with the inclusion material (large arrows) (10 100 x)

Fig 34 *Newborn kitten. Longitudinal section. Microglial inclusion of type III Note the central dense area (3) and the peripheral vesiculated less dense zone (P) (8100 ×)*

Fig. 35 On week old kitten. Longitudinal section. Two microglial cells (MG 1 and MG 2) with large polymorphous inclusions of type III (3) and IV (4) (5800 ×)

Figs. 36—40 are light micrographs and Figs. 41—45 lectron micrographs illustrating Wallerian degeneration in immature spinal cord white matter

Fig. 36 Note the myelinated ligodendrocyt (1) and the nucleated body (2) Compare Figs. 5 a and 9 (1500 ×)

Fig. 37 Two cells containing large vacuoles can be seen (arrows) The lower cell also contains a dense inclusion material. Compare Figs. 5 b, 6 and 7 (1500 ×)

Fig 38. The picture shows a partly myelinated oligodendrocyte (1) a nucleated body (2) and cell with dense inclusions (3) Compare Figs. 5 b, 6 and 10 (1500 ×)

Figs. 36—38 show many collapsed myelin sheaths and myelin-like bodies. Compare Figs. 1 b, 3 and 4

Figs. 39 and 40 show nucleated bodies ( ) Compare Fig. 8. (1500 ×)

Fig. 41 20 day old kitten Longitudinal section. Tw microglial cells (MG 1 and MG 2) containing large polymorphous inclusions. Compare Fig 35 (9100 ×)

Fig 42. 20 day old kitten. Transverse section. Degenerating glial cell. The vesiculated cytoplasm contains many swollen mitochondria (m) Th dense irregular nucleus is indicated by N Compare Fig. 11 (9500 ×) Inset shows microglial inclusion similar to that in Figs. 34 and 35 (6000 ×)

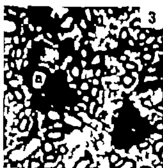
Fig 43 20 day old kitten. Transverse section. A glial cell, possibly an oligodendrocyt (OL) is enclosed by my lin. Compare Fig 13. (8700 ×)

Fig 44 20 day old kitten. Transverse section A glial cell resembling microglia (MG) is enclosed by my lin. (8700 ×)

Fig. 45. Twelve day old kitten Microglial inclusion (N = microglial nucleus) which seems t be composed of folded myelin sheath. (14 100 ×) Inset illustrates the rea ndicated by th rectangle t higher magnification. Compare Fig 31 (113,250 ×)


1a
A

1b

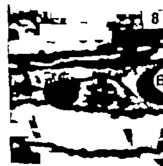
2

3

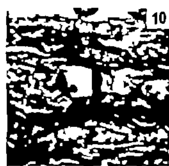
4

5a

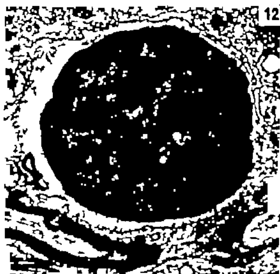
5b

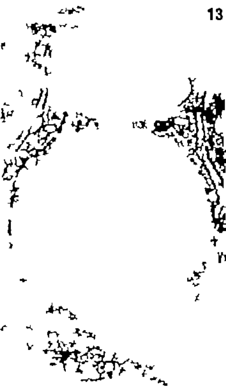
6

7

8
B

9

10


11


12


13


14


15
A


16
A

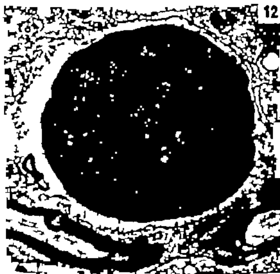

17
A


18
1
2
3
4
B


20

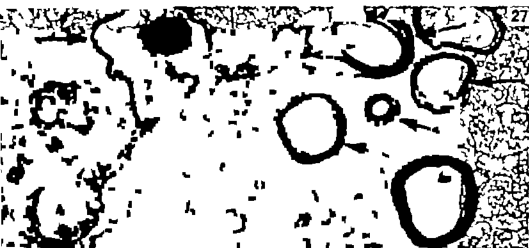

19

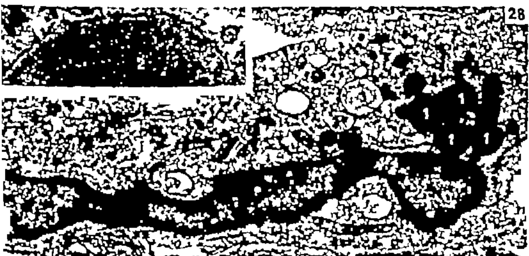

11


12


13


14


27


28
1
1
1
1
M

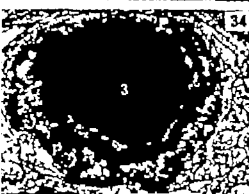

29


30
M


31
MB


32

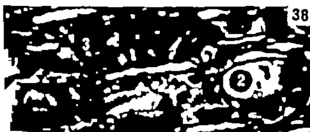

33


34
3


35
3


36
1
2


37


38
3
2

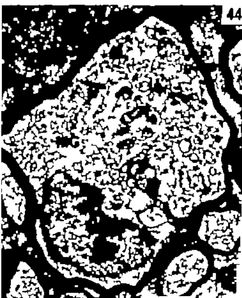

39
n


40
n


41


42


43


44


45

Histochemical studies of adult and developing feline spinal cord white matter

By

Claes Hildebrand and Sten Skoglund

Introduction

Signs of a myelin sheath disintegration and segmental demyelination have been observed light and electron-microscopically in developing peripheral nerve fibres (Berthold and Skoglund 1968 a b) Marchi-positive and OTAN black bodies and granules have later been demonstrated in the paranodes and short internodes of peripheral nerve fibres at the same period of development (Berthold, to be published) After incubation for acid phosphatase the immature peripheral fibres show precipitates indicating the presence of this enzyme in Schwann cell cytoplasmic bodies and around myelin fragments (Berthold, to be published) These histochemical observations thus support the original discovery of a degenerative process affecting immature peripheral myelin sheaths. During the degeneration period the nodal-paranodal region differentiates and attains a nearly mature structure when the fibres reach a diameter around 4 μ (Berthold 1968 a) At this fibre size the myelin sheath becomes crenated paranodally and cords of mitochondrion loaded Schwann cell cytoplasm extend towards the node gap The distribution and postnatal increase in number of paranodal Schwann cell mitochondria has been inves[illegible] gated by means of histochemistry and electron microscopy (Berthold [illegible] Skoglund 1967) In the node gap the number of Schwann cell processes [illegible] ually increases and they become more regularly arranged as devel[illegible] proceeds (Berthold and Skoglund 1967 Berthold 1968 a) The [illegible] morphological changes outlined above has been denoted the [illegible] process (*Berthold 1968 a*)

A nodalization process also seems to occur in developing central [illegible] the feline spinal cord white matter exhibits signs of myelin sheath [illegible] tion and oligodendroglial cell death, interpreted as a breakdown [illegible] of myelin-glial units during the first postnatal weeks. These fea[illegible] described and discussed in a separate paper (Hildebrand 1971 [illegible] period there is a proliferation of microglial cells (cf. Del R[illegible] The perivascular and some interstitial microglial cells conta[illegible]

cytoplasmic bodies. Large polymorphous inclusions are present inside other interstitial microglial cells (Hildebrand 1971 a) It is assumed that these inclusions represent phagocytized myelin fragments and necrotic oligodendroglial cells, the degradation of which is accomplished by lysosomal enzymes.

Degenerating myelin can be demonstrated with the Marchi and OTAN methods (see Adams 1959 1965) and the acid phosphatase reaction can be used for histochemical and cytochemical identification of lysosomes (De Duve 1963 Novikoff 1963 De Duve and Wattiaux 1966) By applying these methods to the developing white matter it should be possible to obtain histochemical evidence for the suggested breakdown of myelin-glial units in the early post natal period.

In mature peripheral nerve fibres the nodal axon is surrounded by an extracellular matrix in which the nodal Schwann cell processes are embedded (cf. Landon and Williams 1963 Berthold 1968 b) Recently Langley and Landon (1969) have elaborated a histochemical method for the demonstration of polyanions which when applied to peripheral nerve fibres specifically demonstrates the node gap substance. In large fibres of adult feline spinal cord white matter the nodal axon is also surrounded by an extracellular matrix through which delicate astrocytic extensions pass to reach the immediate vicinity of the nodal axon membrane (Hildebrand 1971 b) If the matrix substance around the nodal axon of central fibres has a chemical composition similar to that of peripheral fibres it should give a positive reaction with the histochemical method of Langley and Landon, at least in adult animals.

In developing future large central fibres the adult nodal ultrastructural pattern is not established until the fibres have reached a diameter around 5 μ and below that size only small amounts of extracellular material can be seen electron-microscopically at the nodes of Ranvier (Hildebrand 1971 c) It would thus be of interest to know whether the node gap substance is histochemically demonstrable in immature central fibres. This has not been investigated in developing peripheral nerve fibres.

The present paper is an attempt to answer the following questions

1 Are there any Marchi-positive and OTAN-black structures in normal white matter of developing and adult cats?
2. Is there a positive reaction for acid phosphatase in the microglial cytoplasmic bodies and inclusions in the early postnatal period?
3 Does the method of Langley and Landon (1969) for the demonstration of polyanions in peripheral nerves stain the extracellular nodal matrix of adult and developing central fibres?

Material and methods

9 adult cats and 33 kittens were fixed by vascular perfusion. The fixative was administered by means of a teflon cannula inserted into the ascending aorta through an incision in the left ventricle. At the onset of perfusion the right atrium was cut open. During the operation artificial respiration with 100 % oxygen was used. After a short rinse with a Ringer or Tyrode solution containing 2.7 % Rheomacrodex (Pharmacia, Sweden) the fixative was perfused at a controlled intermittent pressure (Berthold 1968 c). The fixative consisted of 5 % glutaraldehyde in a Millonig or cacodylate buffer and also contained 2.7 % Rheomacrodex. The glutaraldehyde was always purified before use by shaking it with active charcoal or by distillation (Fahimi and Drochmanns 1968).

After perfusion the first two cervical spinal cord segments were removed. In older kittens and adult cats the lateral funiculi were cut free and trimmed down to smaller pieces. In the youngest kittens the transversely sliced spinal cord segments used were only divided by a median cut. The pieces of white matter were usually postfixed for 10—120 minutes in the perfusate, lacking Rheomacrodex, followed by a rinse in the buffer used. Some of the specimens to be incubated for acid phosphatase were directly brought to the rinsing solution without postfixation. From this point on three different methods were employed.

1. To produce specimens for the Marchi and OTAN reactions the glutaraldehyde was dissolved in cacodylate or Millonig buffer. Earlier workers used formalin fixation (see Adams 1965) but it is known from studies of peripheral nerves that glutaraldehyde fixation renders comparable results (Berthold, personal communication). Non frozen sections (20—30 μ) cut with a Smith-Farquhar tissue sectioner (Sorvall, USA) were immersed for 18—24 hr [illegible] a mixture of 1 part 1 % osmium tetroxide and 3 parts 1 % $KClO_3$ [illegible] in distilled water. This step represents the Swank Davenport modifi[illegible] Marchi staining (see Adams 1965). Some sections from each experiment [illegible] mounted in glycerine without further treatment. Other sections were [illegible] in a saturated solution of alpha-naphtylamine at 37° C for 1—2 [illegible] (Adams 1965) rinsed and mounted in glycerine. In a few instances [illegible] frozen sections were immersed in acetone at 4 C for 10 minutes [illegible] Marchi and OTAN staining. Frozen sections (10 μ) were also [illegible] Adams 1965) but rendered inconclusive results since most of the [illegible] tive material which was observed in non frozen sections appeared [illegible] well after freezing (see Discussion). Exposing the sections to a [illegible] amine for 1—2 minutes instead of the 1—20 minutes recommended [illegible]

original description (see Adams 1965) was found to be preferable because the weaker staining of normal myelin then obtained gave a better contrast to the black Marchi-positive material

2 The localization of acid phosphatase activity was determined on specimens from animals fixed by glutaraldehyde in the cacodylate buffer Sections cut with the tissue sectioner (?0—30 μ) were incubated in a freshly preparated Gomori medium (Gomori 1952) with sodium-β-glycerophosphate (0 01 M) as substrate and lead nitrate (0 004 M) as reagent dissolved in an acetate buffer (0 05 M) at pH 5 The control sections were incubated in a medium lacking the substrate or containing 0 01 M NaF After incubation at 37° C for 15 30 45 and 60 minutes the sections were rinsed in the acetate buffer developed" for 1—? minutes in 1 % ammoniumsulfide, rinsed again and mounted in glycerine for light microscopy In the few instances when frozen sections (10 μ) were used the same procedure was followed. After incubation and rinse some of the non-frozen sections were mordanted 1—2 hours in 2 % osmium tetroxide in a veronal-acetate buffer (Palade 1952) After a rinse these sections were rapidly dehydrated in a graded series of acetone solutions and embedded in Vestopal W (Ryter and Kellenberger 1958) for ultrastructural localization of the reaction product. The thin sections were examined in a Philips EM 300 electron microscope.

3 For demonstration of the nodal extracellular matrix specimens were taken from animals fixed by glutaraldehyde in Millonig buffer Langley and Landon (1969) used teased peripheral nerves for the demonstration of polyanions in the node gap but since teasing is not convenient in case of white matter another technique was used. The pieces of white matter were cut into 100 μ thick sections with the tissue sectioner and immersed for two hours in 2 % osmium tetroxide in the Millonig buffer After a short rinse in the buffer the sections were incubated for 1 hour in a solution of copper sulphate (0 004 M) and sodium citrate (0 0034 M) in a 0 05 M phosphate buffer at pH 5 Subsequently the specimens were rinsed for 15 minutes in the phosphate buffer and transferred to a solution of potassium ferrocyanide (0.01 M) acidified to pH 1.8 by addition of HCl. For further details on the composition of the solutions see Langley and Landon (1969) Thirty minutes later the specimens were rinsed for 15 minutes in the buffer and, after a rapid ethanol dehydration, embedded in paraffin. Sections were cut at a thickness of 5 μ and mounted for light microscopy In a few instances frozen sections were incubated as described above and mounted in glycerine. However the frozen sections never showed any precipitates (cf. Zenker 1969)

For the light microscopy a Leitz Wetzlar photomicroscope supplied with Kodak Panatomic X rollfilm was employed

Results

1 The Marchi and OTAN reactions

In longitudinal non frozen sections from the lateral funiculus of kittens less than three weeks old the myelin sheaths frequently showed some irregularities (cf Hildebrand 1971 a) These occurred either as single outgrowths or as clusters of evaginations with a paranodal or internodal position (Figs. 1 and 3) Along the myelinated fibres rounded globules, which like the myelin sheaths were Marchi-negative and OTAN red, could be seen (Figs. 4 and 5) Furthermore a number of Marchi-positive and OTAN-black bodies of varying sizes were noted (Figs. 2—4 and 6—8) Some of these appeared to be homogeneously black but often a lighter halo or light center was present Frequently the Marchi-positive and OTAN-black bodies had an irregular shape resembling twisted myelin sheaths (Figs. 3 b and 7) Other bodies showed an elongated ovoid or spherical shape The black bodies seemed to be most common along the larger myelinated fibres. In addition to the black bodies intracellular clusters of small Marchi-positive and OTAN-black granules were observed between the myelinated fibres and close to blood vessels (Fig 9)

The intracellular clusters of small Marchi-positive and OTAN-black granules were common during the first three postnatal weeks but rare or absent in the adult The Marchi-positive and OTAN black bodies appeared to increase greatly in number during the first three postnatal weeks. In sections from the lateral funiculus of kittens older than three weeks and from adult animals the bodies were numerous and arranged in rows between the myelinated fibres (Figs. 10 and 11) The black bodies seemed to be larger and more compact in the adult than in the early postnatal stage. After the third post natal week most of the black bodies were seen close to the nodes of large fibres, particularly in adult animals (Figs. 12 and 13) They were located outside the paranodal myelin sheaths at varying distances from the node gap This arrangement was very similar to that of the myelinoid bodies described in an earlier report on the morphology of the large central nodes of Ranvier (Hildebrand 1971 b see also Hildebrand 1971 c)

On the basis of the size, shape and distribution of the Marchi-positive and OTAN-black bodies and in particular their presence at the nodes of Ranvier it is concluded that they correspond to the lamellated globules previously

denoted as myelinoid bodies (Hildebrand 1971 a, b c) The intracellular clusters of black granules might represent lipid inclusions or possibly lysosomes (cf. Elleder and Lojda 1968 a, b Lojda and Elleder 1968 Andrews and Kruger 1969)

Sections subjected to acetone extraction before staining did not show any Marchi positive or OTAN black material. The myelin sheaths, however remained OTAN red.

The description above refers to the non-frozen sections. The frozen sections rendered inconclusive results with the Marchi and OTAN methods. Marchi-positive and OTAN-black bodies were seen in a few instances but as a rule the bodies which could be discerned had a grayish colour when Marchi stained (Fig 14) and were brown after the OTAN reaction.

2. The acid phosphatase activity

Light microscopy

In animals less than three weeks old both the superficial and deep parts of the lateral funiculus showed a high incidence of precipitates indicating acid phosphatase activity (Figs. 15—20) The reaction product appeared as a dark brown staining of granules and globules varying in size. Many perivascular cells contained groups of strongly positive small granules (Fig 15) and numerous similar granules occurred interstitially either singly or in clusters (Figs. 16 and 17) The reaction product was also present in larger compact globules which appeared to represent glial inclusions (Figs. 18 and 20) Furthermore the activity was sometimes arranged in rings or was crescentshaped surrounding translucent globules of varying sizes (Figs. 19 and 21)

In the superficial part of the lateral funiculus a reduction in number of positive granules and globules was discernible from the third week on. In the deepest part of the lateral funsculus the reduction was less evident. The activity of acid phosphatase, present as granules in perivascular cells and as scattered interstitial granules, was sparse in the superficial part of the adult lateral funiculus. Close to the large myelinated fibres the reaction product was in many instances arranged as a thin halo around what appeared to be myelinoid bodies (Figs. 22 23 24 and 25) (Hildebrand 1971 b) The myelin sheaths were unstained and barely visible in these sections. By varying the focus it was sometimes possible to note the presence of a node of Ranvier in the immediate neighbourhood of the rings of precipitates (Fig 25)

The neurons in the ventral horn were loaded with positive granules at all developmental stages and several perivascular cells in the gray matter remained positive throughout the postnatal period. Consequently the gray matter

could be used as a check that the method worked even when the white matter showed very little reaction products.

No activity was seen in the controls. The non-frozen sections were superior to the frozen sections with respect to general tissue preservation and also showed a somewhat stronger staining. The most distinct localization of the reaction product was obtained with an incubation time of 30—45 minutes. A longer time in the incubation medium increased the incidence of artefacts such as nuclear staining and a shorter time (15 minutes or less) resulted in faint activity.

Electron microscopy

In order to obtain a more precise localization of the reaction product in immature white matter thin sections from the superficial part of the lateral funiculus of kittens less than three weeks old were examined in the electron microscope. The thin sections were stained with uranyl acetate or unstained. Dense finely granular lead deposits were located in the lysosome-like cytoplasmic bodies of perivascular and interstitial microglial cells (Figs. 26 and 27) and in oligodendroglial dense bodies (Figs. 30, 31 and 32). Lead deposits were also present within microglial inclusions (Fig. 29). Deposits of the reaction product surrounded and were contained within various lamellated bodies some of which showed a lamellar pattern similar to that of normal myelin or myelinoid bodies (Figs. 34—37) (Hildebrand 1971 b).

Often some fine general cytoplasmic precipitates were noted and in the myelin sheaths, mainly along the minor dense lines, small amounts of lead precipitates could be seen. Since this also occurred in the controls, as did reaction products associated with the luminal membrane of endothelial cells, it represents unspecific deposits of metal. The glial nuclei, particularly those of microglia, often contained precipitates (Fig. 29). This localization is likely to be an artefact (cf. Maunsbach 1966). In the perivascular microglia the cytoplasm was frequently loaded with the reaction product whereas the cytoplasmic bodies in that case only contained a faint precipitate or none at all (Fig. 28). This is interpreted as an artefact caused by diffusion of the enzyme or the reaction product during preparation of the tissue (cf. Novikoff 1963, Reale and Luciano 1970).

3. The perinodal matrix

In paraffin sections of non-frozen adult white matter processed according to Langley and Landon (1969) a black dense precipitate was present at many nodes. In *transverse sections the precipitate formed a ring around the nodal*

axon (Fig 38) and in longitudinal sections it appeared as a bar across the nodal axon or as two dense dots, one on each side of the nodal axon (Fig 39) This pattern is identical with that in nodes of peripheral nerves described by Langley and Landon (1969) (cf Adams *et al.* 1969 Zenker 1969) Sometimes the reaction product also extended into the decrement zones presenting the picture of a cross of Ranvier" or was limited to the decrement zones. Often the nodal axon contained varying amounts of precipitate (Fig 38 c) (cf Langley and Landon 1969 Zenker 1969) Localization of the reaction product exclusively to the decrement zones and to the axon is likely to be an artefact due to insufficient fixation. It is possible that the presence of some precipitate within the axoplasm and in the region of myelin termination combined with a heavy precipitate in the node gap is not an artefact (Langley and Landon 1969) In the immature animals (10 15 and 25 days) nodes with deposits of copper ferrocyanide were much less frequent as compared to the adult stage. Deposits were seen only at nodes of large fibres (Fig 40) (the smallest positive fibre observed measured around 5 μ) and often appeared less distinct compared to the adult (Fig 40 b)

Discussion

1 The OTAN and Marchi reactions

The myelin breakdown products formed in Wallerian degeneration and demyelinating diseases are stained black with the Marchi method whereas normal myelin is unstained (see Adams 1965) In addition to the heavy black staining of degenerating tracts a variable amount of so called pseudo-Marchi staining in normal white matter is a well known feature generally considered as an artefact (Smith 1956 a)

The Marchi-positive material during early Wallerian degeneration differs from that at later stages (Smith 1956 b Strich 1968) The first month after nerve section the degenerating material appears as black bodies of varying size and identical but less numerous pseudo-Marchi bodies are present in nondegenerating areas Later the degenerating material is redistributed to form intracellular aggregates of Marchi-positive granules (Strich 1968) The early breakdown products and the pseudo-Marchi bodies stained black only when non-frozen blocks of tissue were mordanted in Marchi fluid and not when frozen sections were used (Strich 1968) Similarly the Marchi-positive and OTAN-black bodies observed in the present study did not stain in a clearcut way when frozen sections were used. The late degenerating *material however* is stained also in frozen sections (Strich 1968) Obviously it is not convenient

to use frozen sections for the Marchi reaction since then only the late break down products are seen and consequently the early period of myelin disintegration will be overlooked. It seems to be well established that the late myelin breakdown products to some extent are composed of cholesterol esters (see Adams 1965). The chemical composition of the early Marchi material is probably more complex (cf. Strich 1968).

The OTAN method is a further development of the Marchi reaction. With the introduction of alpha naphtylamine after the Marchi reaction normal myelin is stained red. The theoretical basis for the Marchi and OTAN reactions has been thoroughly discussed by Adams (1965) and the mechanism suggested has gained support by the work of Giolli and Scully (1968). According to Adams unsaturated hydrophobic lipids (triglycerides, cholesterol esters, desmosterol and fatty acids) are stained black with both methods. Unsaturated hydrophilic lipids are stained red with the OTAN method and are not stained by the Marchi fluid. The specificity of the methods has been questioned by some workers (Elleder and Lojda 1968 a, b; Lojda and Elleder 1968; Andrews and Kruger 1969; see also Adams and Bayliss 1968).

Studying the white matter of developing man and chick Adams and Davison (1959) noted that at a certain stage a great deal of the cholesterol was esterified. In the human cervical cord the amount of cholesterol ester reached a peak around the 35-th week of gestation and an ester peak was also noted in the corpus callosum of the 8 week old infant. According to Langworthy (1933) the human cervical cord contains considerable amounts of myelin by 29 weeks in utero and the corpus callosum is partly myelinated 4 weeks after birth. Apparently myelination is well under way when the ester peak appears and therefore the latter is not necessarily related to the initial myelination (cf. Adams 1965). Several other workers likewise have pointed out the presence of esterified cholesterol in the developing central nervous system (Williams *et al.* 1945; Brante 1949; Mandel *et al.* 1949; Johnson *et al.* 1949; Howell *et al.* 1964; Svennerholm 1964; Grafnetter *et al.* 1965).

In histological sections of immature spinal cord white matter of developing man and chick Adams and Davison (1959) observed "lipid droplets", some of which were OTAN-black, around myelinating axons (cf. Mickel and Gilles 1970). Similar observations were made in the central nervous system of foetal sheep Barlow (1969). These reports are consequently supported by the present findings in the kitten. According to Barlow (1969) the "lipid droplets" first stained red with OTAN and contained birefringent elements. Later the "lipid droplets" stained black with OTAN and less frequently showed birefringence. He also stated that the "granular and discontinuous" early myelin sheaths continuously incorporated lipid droplets. The latter view is not compatible

with modern concepts of myelinogenesis (Geren 1954 1964) A transformation of Marchi-negative birefringent elements into a Marchi-positive non-birefringent material suggests a process of myelin disintegration rather than the reverse.

In the developing spinal cord white matter of kittens certain light and electron microscopic features indicating a breakdown of some myelin-glial units have been described (Hildebrand 1971 a) The hypothesis was advanced that developing, future large myelinated central fibres pass through a process of myelin disintegration and segmental demyelination in analogy with developing peripheral myelinated fibres (Berthold and Skoglund 1968 a, b) Against this background it seems reasonable to suggest that the Marchi-positive and OTAN black material in the developing white matter found here represents degenerating myelin

In this connection the results of Pritchard (1963) might be of relevance. According to her part of the newly synthetized cholesterol in the developing rat brain during early myelination is lost somewhat later Only about 50 % of the marked cholesterol in 10 day old animals, formed from labelled precoursors injected shortly after birth, was retained by the age of 40 days. After that age, however the level remained largely constant. These results could be explained in terms of a myelin sheath disintegration affecting some of the newly formed internodes.

It is noteworthy that Grant (1968) in a Nauta study on retrograde neuronal degeneration in kitten spinal cord observed argyrophilic granules along myelinated fibres in the controls. As suggested by him this might indicate a normally occurring degenerative process affecting immature myelinated central fibres.

In an earlier report (Hildebrand 1971 b) the lamellar spacing of myelinoid bodies in adult white matter was found to be about 45—50 A. Lamellated bodies or masses with a similar period were common during the early stage of Wallerian degeneration (Hildebrand 1971 a) Thus it seems that the normally occurring myelinoid bodies resemble the myelin breakdown products in the early stage of Wallerian degeneration. Furthermore it appears very likely that the myelinoid bodies correspond to the pseudo-Marchi bodies noted by others in non-degenerating adult white matter (see Smith 1956 a, Strich 1968) In the present study the pseudo-Marchi" positivity of adult white matter was located in a distinct structure, the myelinoid body and the myelin sheaths were never stained black. Therefore the Marchi-positivity in normal white matter with the configuration described above should not be considered as an artefact. Unspecific black precipitates did occur but they were few and easily distinguishable from myelinoid bodies.

No Marchi-positive or OTAN black bodies were seen in sections subjected

to a short actone extraction whereas the myelin sheaths remained OTAN red This observation favours the idea that the black bodies are chemically similar to degenerating myelin since acetone extracts mainly hydrophobic lipids such as cholesterol esters (Elleder and Lojda 1968 b Adams and Bayliss 1968 b) As stated above the Marchi-positive material in degenerating myelin is probably esterified cholesterol (Adams 1963) Accordingly small amounts of cholesterol esters have been found in normal adult white matter (for references see Fewster *et al.* 1970) However some earlier workers were unable to detect any cholesterol ester in mature white matter (Adams and Davison 1959 1960) This contradiction might be accounted for by the different isolation and extraction procedures. Since the Marchi-positive and OTAN-black bodies prevailed along the large myelinated fibres it also seems that the selection of anatomical areas to be analyzed is of importance (cf Discussion in Adams and Davison 1959)

It is well known that in Wallerian degeneration of central fibre tracts the Marchi-positive material persists for a very long time after nerve section in contrast to the fairly rapid removal of degenerating myelin in peripheral nerves (see Strich 1968 Daniel and Strich 1969) Accordingly the lipid loss in degeneration proceeds at a higher rate in peripheral nerves than in central fibre tracts although the pattern of lipid depletion is qualitatively similar (McCaman and Robins 1959 a) These histochemical and chemical results are concordant with electron microscopic evidence that in the central nervous system the removal of myelin débris during Wallerian degeneration is a very slow process as compared to that in peripheral nerves (Luse 1960 Bignami and Ralston 1969 Kruger and Maxwell 1969 Vaughn *et al* 1970)

The slow removal of degenerating myelin from central tracts might then account for the presence of Marchi-positive and OTAN black bodies also in older kittens and adult cats. In that case the black material would constitute accumulated remnants from an early period of myelin disintegration. On the other hand a gradual removal of the early postnatally formed myelin break down products would suit better with the results of Pritchard (1963) cited above. The latter view is supported by the presence of many microglial phagocytes in immature white matter (Del Río-Hortega 1932) Furthermore intra cellular clusters of small Marchi-positive granules resembling those observed in late Wallerian degeneration (Smith 1956 b Strich 1968) were present in the immature animals. Finally it would be difficult to reconcile the thought that all the adult black bodies are remnants of an early myelin sheath disintegration with the fact that in the adult the Marchi positive and OTAN-black bodies are mainly located to the paranodes of large fibres and also seem to be larger and more numerous than in the early postnatal stage.

However there might be two separate processes in operation, which overlap in time. The disintegrating myelin of the most immature animals is likely to be at least partly removed by proliferating microglia (cf. Del Rio-Hortega 1932) The presence of myelin breakdown products in juvenile and adult white matter could then be explained in terms of a continuous slow myelin disintegration by formation of paranodal myelin buds which detach from their origin ((cf. Hildebrand 1971 c) and degenerate Such a continuous loss of myelin material must necessarily be compensated for by generation of newly myelin at other sites and this would be reflected as a slow turnover of myelin constituents which actually takes place (see Davison and Gregson 1966 Smith 1967 1968 1969)

In the present work the occurrence of black bodies was not quantitatively assessed for technical reasons. The statements made of an increase in number of such bodies during the first postnatal weeks are founded on the subjective impression from inspecting the sections. A quantitative approach relating the number of bodies to the increasing number and size of myelinated fibres will have to await an improved methodology

2. The acid phosphatase activity

Before the third postnatal week the incidence of precipitates indicating acid phosphatase activity was high in the spinal cord white matter and thereafter subsided to a low level in the adult. Other workers have also reported higher levels of acid phosphatase in immature than in adult central nervous tissue (Flexner and Flexner 1948 Flexner 1955 Cohn and Richter 1956 Wender *et al* 1970)

In the immature white matter the lead deposits formed during incubation were mainly located in microglial lysosome like bodies, microglial inclusions, oligodendroglial dense bodies and around and within myelinoid bodies, myelin fragments and other lamellated bodies (cf Hildebrand 1971 a) The presence of precipitates in the microglial cytoplasmic bodies together with their ultra structural appearance (Hildebrand 1971 a) support the view that these bodies are lysosomes (De Duve 1963 Novikoff 1963 De Duve and Wattiaux 1966)

It is well established that phagocytes in general contain lysosomes (Cohn *et al.* 1963) and that in the central nervous system the microglia may serve as phagocytizing elements (Blinzinger and Hager 1964 Hager 1968) Lysosomal enzymes play an important role in degradative processes within cells and during the process of phagocytosis lysosomal enzymes are discharged into phagocytic vacuoles (De Duve 1963 Cohn *et al.* 1963) A proliferation of phagocytic cells with increased activity of acid phosphatase also accompanies

the myelin sheath disintegration in Wallerian degeneration as well as various demyelinating conditions in central nervous tissue (Roizin and Kolb 1957 McCaman and Robins 1959 b De Sibrik and O Doherty 1960 Friede 1961 1968, Adams 1962, Nelson *et al* 1962, Resibois 1969 Vaughn *et al* 1970)

The formation of precipitates in immature white matter around and within myelinoid bodies and myelin fragments thus may indicate a process of degradation, although a clearcut relationship to a microglial cell could not always be observed Possibly oligodendrocytes may contribute to the breakdown of myelin fragments and myelinoid bodies since their dense bodies also contained precipitates.

The present findings together with the occurrence of Marchi positive and OTAN-black bodies in developing white matter support the proposal advanced in an earlier study (Hildebrand 1971 a) that during an early stage of white matter development a number of myelin-glial units disintegrate and become engulfed by microglial phagocytes. Within the microglial cells the degradation of the phagocytized material would then take place with the aid of lysosomal enzymes.

The adult white matter contained very little reaction products after incubation for acid phosphatase (cf Shanta *et al* 1967 Manocha 1969) In adult animals part of the reaction product appeared to be located around myelinoid bodies. This feature favours the assumption (see above) that myelinoid bodies are continuously formed and broken down in the adult white matter thereby possibly subserving the turnover of myelin material in mature sheaths (Davison and Gregson 1966, Smith 1967 1968, 1969)

3. The perinodal matrix

The histochemical method used by Langley and Landon (1969) for demonstration of the extracellular node gap substance in peripheral nerve fibres also stained the central nodal matrix. The light microscopic location and configuration of the reaction product in central nodes was identical with that in peripheral nodes (Adams *et al.* 1969 Langley and Landon 1969 Zenker 1969) No reaction product was found in the frozen sections. As reported by Zenker (1969) the stainability of peripheral nodes is also greatly diminished by freezing.

The fact that both the central and the peripheral nodal matrix can be visualized with the same reagents suggests that they are chemically similar This favours the assumption that the two substances are playing an analogous functional role. According to Langley (1969) the peripheral nodal matrix acts as a cationic exchange resin being present in comparatively large amounts at

those sites of myelinated fibres where the ion exchange takes place during impulse propagation.

In the immature white matter only a few nodes showed precipitates and the positive nodes observed always belonged to large fibres. This finding is in accordance with previous ultrastructural studies of developing central nodes of Ranvier indicating that immature fibres with a diameter less than 4—5 μ possess only small amounts of nodal extracellular matrix (Hildebrand 1971 c). In nodes of large adult central fibres the nodal axon is surrounded by specialized astrocytic extensions which are embedded in the nodal matrix (Hildebrand 1971 b). Possibly the nodal matrix is produced and maintained by the nodal astrocytic processes (cf Lumsden 1968, Dorfman and Ho 1970). It is then tempting to correlate the sparse occurrence of extracellular substance at nodes of immature central fibres smaller than 5 μ with the absence or weak development of astrocytic processes at these nodes (see Hildebrand 1971 c, cf Hess 1955).

In conclusion the studies with the Marchi and OTAN methods, the incubation for acid phosphatase and the staining of the nodal extracellular matrix confirm and supplement earlier ultrastructural and light microscopic results (Hildebrand 1971 a, b and c). Apparently the nodalization concept, which was founded on studies of developing peripheral nerves (see Berthold 1968 a), may be extended also to developing central fibres and probably the same functional correlations can be applied.

Summary

Earlier studies indicated that immature, future large myelinated fibres in feline spinal cord white matter underwent a degeneration of myelin-glial units at an early stage of development. This was followed by nodal differentiation. To obtain histochemical information on these processes, developing and adult spinal cord white matter was investigated with respect to 1. the Marchi and OTAN reactions, 2. incubation for acid phosphatase and 3. staining of the node gap substance.

The immature white matter showed Marchi positive and OTAN-black bodies, which increased in number during the first three postnatal weeks and were numerous in juvenile and adult cats. Intracellular Marchi-positive and OTAN-black granules were present in the immature white matter but rare or absent in the adult. Both in immature and mature white matter the paranodal myelinoid bodies were Marchi positive and OTAN black.

The acid phosphatase activity was most conspicuous during the first three

postnatal weeks being located to microglial cytoplasmic bodies and inclusions, oligodendroglial dense bodies and inside or around various lamellated bodies. After the third postnatal week the incidence of reaction products decreased to a low level in the adult in which some of the precipitates seemed to be located around myelinoid bodies.

In adult white matter the node gap substance showed a stainability and distribution as in peripheral nerves. In kittens only a few large fibres showed staining of the nodes.

The results support earlier presented evidence that a nodalization process occurs in central myelinated fibres

Acknowledgements

This investigation was supported by grants from Karolinska Institutet (Reservationsanslaget) and from the Swedish Medical Research Council (projects no. B70-14X 136-06 and B71 14X 136-07A) We are much indebted t Miss Anita Bygren and Mrs. Ingegerd Lindqvist for skilful technical assistance.

References

ADAMS C. W. M. A histochemcial method for the simultaneous demonstration of normal and degenerating myelin. *J Path Bact* 77 648—650 (1959)

ADAMS, C. W. M. The histochemistry of the myelin sheath. I *Neurochemistry* (K. A C. Elliot, I H. Page and J H. Quastel eds.) Thomas, Springfield, pp. 85—112 (1962)

ADAMS, C. W M. Neurohistochemistry Elsevier Amsterdam (1965)

ADAMS, C. W M. and BAYLISS, O Reappraisal of osmium tetroxid and OTAN histochemical reactions. *Histochemie* 16 162—166. (1968)

ADAMS, C. W M. and BAYLISS, O Histochemistry of myelin. IV Solvent action of acetone on brain and other lipid-rich tissues. *J Histochem. Cytochem.* 16 115—118 (1968 b)

ADAMS, C. W M BAYLISS, O B and GRANT R. T Copper binding and cholinesterase activity around the node of Ranvier *J Histochem Cytochem* 17 125—127 (1969)

ADAMS, C. W M. and D VISON A. N The occurrence of esterified cholesterol in th developing nervous system. *J N urochem.* 4 282—289 (1959)

ADAMS, C. W M. and D son, A. N The form in which cholesterol occurs in the adult central nervous system. *J Neurochem.* 5 293—296 (1960)

ARMSTRONG, J M. and KRÜCKE, L. The distribution of Marchi granules in thalamic degeneration. *Brain Res* 15 537—541 (1969)

BARLOW R. M. The foetal sheep Morphogenesis of the nervous system and histochemical aspects of myelination. *J Comp. Neurol.* 135 249—262 (1969)

KRUGER, L. and MAXWELL, D S Wallerian degeneration in the optic nerv of reptile. An electron microscopic study *Amer J Anat 125* 247—270 (1969)

LANDON, D N and WILLIAMS, P L. Ultrastructure of the node of Ranvier N tu 199 575—577 (1963)

LANGLEY O K Ion ex hange t the nod of Ranvier *Histochem. J 1* 295—309 (1969)

LANGLEY O K. and LANDON D N Copper binding t nodes of Ranvier: a new electron histochemical technique for the demonstration of polyanions. *J Histochem. Cytochem. 17* 66—69 (1969)

LANGWORTHY O R. Development of behaviour patterns and myelinization of the nervous system in the human fetus and infant. *C gie Inst Contrib Embryol* 24 1—57 (1933)

LOJDA, Z. and ELLEDER M. Comments about the paper C. W M. Adams and O B. Bayliss, Reappraisal of osmium tetroxide and OTAN histochemical reactions. *Histochemie 16* 167—169 (1968)

LUMSDEN, C. E. Nervous tissue in culture. In *Th structu e and Fun tion f Nervous Tiss I Str ctur* (E. H. Bourne ed.) Academic Press, New York, pp. 67—140 (1968)

LUSE, S. A. Experimental demyelination. An electron microscopic study *An t Rec 136* 236—237 (1960)

MANDEL, P BIETH R. and STOLL, R. La repartition des diverses fractions lipidique dans le cervea de l'embry de poulet durant la seconde partie de l'incubation. *C R. Soc Biol.* (Paris) *143* 1224—1226 (1949)

MAROCHA, S L. Histochemical distribution of alkaline and acid phosphatase and ATP ase in the brain of squirrel monkey *Histochemie 21* 221—235 (1970)

MAUNSBACH, A. B Observations on th ultrastructure and acid phosphatase activity of the cytoplasmic bodies in the rat kidney proximal tubule cells. *J Ultrastruct Res 16* 197—238 (1966)

McCAMAN, R. E. and ROBINS, E. Quantitative biochemical studies of Wallerian degeneration in th peripheral and central nervous systems — I. Chemical constituents. *J Neurochem. 5* 18—31 (1959)

McCAMAN, R. E. and ROBINS, E. Quantitative biochemical studies of Wallerian de generation in the peripheral and central nervous systems — I. Chemical constituents. *J Neu ochem. 5* 32—42 (1959 b)

MICKEL, H. S. and GILLES, F H. Changes in glial cells during human telencephalic myelinogenesis. *Brain 93* 337—346 (1970)

NELSON E OSTERBERG, K., BLAW M STORY J and KOZAK, P Electron microscopic and histochemical studies in diffuse sclerosis (sudanophilic type) *Neu ology* (Minneap.) *12* 896—909 (1962)

NOVIKOFF A. B. Lysosomes in the physiology and pathology of cells contributions of staining methods. In *Ciba Found tion Symposium on Ly osomes* (A. V S d Reuck and M. P Cameron eds.) Churchill, London, pp. 36—77 (1963)

PALADE, G E. A study of fixation for electron microscopy *J Exp. Med 95* 285—297 (1952)

PRITCHARD E. T The formation of phospholipids from [14]C-labelled precursors in developing rat brain in vivo. *J Neu ochem 10* 495—502 (1963)

REALE, E. and LUCIANO L. Fixierung mit Aldehyden. Ihre Eignung für histologische und histochemische U tersuchungen in der Licht und Elektronenmikroskopie *Histochemie 23* 144—170 (1970)

RESIBOIS A. Electron microscopic study of metachromatic leucodystrophy III Lysosomal nature of the inclusions. *Act Neu opath* (Berlin) *13* 149—156 (1969)

ROIZIN L. and KOLB, L. C. Neuropathologic relationship of m ltiple sclerosis t the experimental allergic encephalomyelitides (Comparativ histologic and some histochemical tudies) *III Congr Internat N u path* (Acta Med. Belg) Bruxelles pp 57—84 (1957)

RYTER, A. and KELLENBERGER, E. L inclusion au polyester pour l'ultramicrotomie *J Ultrastru t R s. 2* 200—214 (1958)

SHANTA, T R., IIJIMA, K. and BOURNE K. Histochemical studies on the cerebellum of squirrel monkey *A ta Histochem. 27* 129—162 (1967)

SMITH, M C. The recognition and prevention of artefact of the Marchi method. *J Neurol Neurosu g Psychiat. 19* 74—83 (1956 a)

SMITH, M. C. Observations on the extended use of the Mar hi method. *J Neurol Neu osu g Psychiat. 19* 67—73 (1956 b)

SMITH, M. E. The metabolism of myelin lipids. *Adv Lip Res. 5* 241—278 (1967)

SMITH M. E. The turnover of my lin in the ad lt rat. *Biochim Bi phy Act 164* 285—293 (1968)

SMITH, M. E. An in itro system for the study of myelin synthesis. *J Neurochem 16* 83—92 (1969)

STRICH S. J Notes on the Marchi method for taining degenerating myelin in the peripheral and central nervous system. *J N urol. Neu surg Psy hiat 31* 110—114 (1968)

SVENNERHOLM L. The distribution of lipids in the human nervous system. I Analytical procedure. Lipids of foetal and newborn brain. *J Neu ochem. 11* 839—853 (1964)

VAUGHN J E., HINDS, P L. and SKOFF R. P Electron microscopic studies of Wallerian degeneration in the rat optic nerve. I. The multipotential glia. *J Comp. Neurol. 140* 175—206 (1970)

WENDER, M., KOZIK, M and WOJCIECHOWSKI, T Enzymhistochemische U tersuchungen zur Entwicklung der Neuroglia des menschlichen Gehirns. *Act Histochem 36* 32—43 (1970)

WILLIAMS, H. H., GALBRAITH, H KAUCHER, M., MOYER, E. Z RICHARDS, A. J and MACY I G The effect of growth on the lipid composition of rat tissues. *J Biol. Chem 161* 475—484 (1945)

ZENKER, W Cholinesterase nd M tallophilie an den Ranvierschen Schnürringen. *A ta Histochem 33* 247—273 (1969)

Legends to figures

Figs. 1—14 Light micrographs of Marchi- or OTAN-stained non frozen (1—13) and frozen (14) sections.

Fig 1 Marchi. Three day old kitten. Small myelinated fibre with paranodal myelin sheath outgrowths (small arrows) Large arrow points to node of Ranvier (1500 ×)

Fig. 2. OTAN Three day old kitten. Group of OTAN-black bodies (arrow) close to large myelinated fibre (×) (1500 ×)

Fig 3 a and b OTAN Three day old kitten. Nodal region of large fibre at different depths of focus. Fig. 3) Large arrow points to the node gap. Myelin sheath out growths can be seen in the right paranode (small arrows) Fig. 3 b) Large arrow points to the nod gap. Two OTAN black bodies are seen outside the right paranodal myelin sheath (small arrows) (1500 ×)

Fig 4 OTAN Three day old kitten. Between the two large myelinated fibres (×) a group of bodies can be seen. One of these is OTAN-black (arrow) The other appear less intensely tained. (1500 ×)

Fig 5 Marchi. Thirteen day old kitten. Arrow points to structure which resembles con oluted myelin sheath and which is Marchi-negative. (1500 ×)

Fig 6. *OTAN Nine day old kitten. Large myelinated fibre in gray matter Small arrows* indicate incisure of Schmidt Lanterman outsid which two small OTAN-black bodies can be seen. (1500 ×)

Fig. 7 Marchi. Nine day old kitten. Cross section showing large Marchi-positive body (arrow) surrounded by myelinated fibres. (1500 ×)

Fig. 8. Marchi. Thirteen day old kitten. The picture shows a very large Marchi-positive body (arrow) (1500 ×)

Fig. 9 OTAN Thirteen day old kitten. A cell containing numerous OTAN-black granules (arrow) is located close to a blood vessel (B) (1500 ×)

Fig 10. Marchi. Four week old kitten. Clusters of Marchi-positive bodies (arrows) (1000 ×)

Fig 11 Marchi. Four week old kitten. Survey picture to show th high incidence of Marchi-positive bodies in white matter of kittens older than three weeks. The picture was similar in adult cats. (?00 ×)

Fig 12 and b. Marchi. *Adult cat. Nodal region of large fibre at different magnifica* tions. Large arrows indicate node gap. N te the large paranodal Marchi-positive bodies. (a. 1500 × b. 640 ×)

Fig 13 OTAN Adult cat. Nodal region of large fibre (× indicates node gap) Two large OTAN-black bodies are present close t the node. (1500 ×)

Fig. 14 Marchi. Ad lt cat Frozen section. Nodal region of large fibre. Node gap is indicated by × Arrow points to juxtanodal body which showed grayish colour (1500 ×)

Fig. 15—37 show non-frozen (except Fig. 25) sections of whit matter incubated for acid phosphatase Figs. 15—25 are light micrographs and Figs. 26—27 electron micrographs.

Fig. 15. Three day old kitten. Positiv granules in perivascular cell. B = blood vessel. (1500 ×)

Fig. 16 Three day old kitten. Glial cell (N = nucleus) with a negative inclusion (arrow) and several small positiv granules. (1500 ×)

Fig. 17 Three day old kitten. Survey photograph. Note the high incidence of positiv granules. (1500 ×)

Fig. 18. Three day old kitten. Glial cell (nucleus indicated by N) showing a large positi e inclusion (arrow) (1500 ×)

Fig. 19 Three day old kitten. The precipitat indicated by an arrow surround two rounded translucent globules. (1500 ×)

Fig. 20. Thirteen day old kitten. Glial cell contai ing large positiv inclusions (arrow) (1500 ×)

Fig. 21 Three week old kitten. The precipit te indicated by an arrow surrounds tw translucent globules. (1500 ×)

Fig. 22. Adult cat. Precipitate present as small granules and partly surrounding large rounded globule (arrow) (1500 ×)

Fig. 23 Adult cat Ringshaped deposit of reaction product (×) (1500 ×)

Fig. 24 Ad lt cat. Group of rounded translucent globules (×) urrounded by precipitate. Compare Fig 12 b. (1500 ×)

Fig 25 Adult cat. Nodal region. Frozen section. Arrow points t the node Paranodally granular and ringshaped deposits of reaction product can be seen. (1500 ×)

Fig. 26 Eighteen day old kitten. Microglial process with two acid phosphatase-positive bodies (arrows) (1500 ×)

Fig 27 Eighteen day old kitten. Precipitates in cytoplasmic bodies (arrows) of perivascular microglia (B = blood vessel E = endothelial cell) The section passed tangentially through the vessel. (34,500 ×)
Inset.
Eighteen day old kitten. Positiv cytoplasmic body (arrow) in perivascula microglial process (B = blood essel E = endothelial cell) (85,500 ×)

Fig. 28. Eighteen day old kitten. Perivascular microglia with massive deposits of reaction product in the cytoplasm (B = blood vessel) The cytoplasmic bodies (×) contain only faint remnants of precipitat This was common artefact. (18,300 ×)

Fig. 29 Eight day old k tten Microglial inclusion (arrows) with coarse precipitates. No precipitates were se the same typ of inclusion in control specimens. The microglial nucleus (N) con ains some recation product. (7 000 ×)

Fig 30. Eighteen day old kitten. Oligodendroglial process (OP) with three positive cytoplasmic bodies arrows) (36 000 ×)

Fig. 31 Eight day old kitten. High magnification of oligodendroglial acid phosphatase-positive cytoplasmic body Note the membrane rrows) (113,250 ×)

Fig. 32. Eighteen d y old kitten. Acid phosphatase-positive oligodendroglial cytoplasmic body Arrow points to the membrane. (69,500 ×)

Fig. 33 a. Eight day ld kitten. Large acid phosphatase-positive body probably of oligodendroglial origin, in close association with a myelin sheath (M) (88,700 ×)

Fig. 33 b. The same area as in Fig 33 a at a lower magnification. Note the nucleus (N) in th lower half of the picture. (13 400 ×)

Fig 34 Eight day old kitten Deposits of reaction prod ct (arrows) within and around lamellated body (myelinoid body) Note the lamellar pattern which differs from that in normal my lin. (88,700 ×)

Fig 35 Eight day old kitten. Lamellated body containing precipitates. (arrows) The lamellar pattern is similar to that of myelin. (88,700 ×)

Fig 36. Eight day old kitten. The picture shows glial inclusion mainly composed of myelin. Precipitates indicating acid phosphatase activity are associated with th myelin loops (arrow) (8100 ×)

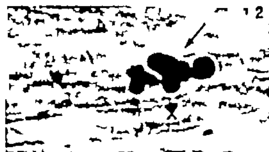
Fig. 37 and b. Eight day old kitten. The pictures show lamellated intracellular bodies which contain th reaction product. (54 600 × b 64,300 ×)

Figs. 38—40 are light micrographs of sections from spinal cord white matter tained for the nod gap substance.

Fig. 38 a, b and c. Transverse sections. Adult cat. I each of th pictures cross-sectioned node surrounded by a dense ring f reaction prod ct can be seen (arrows) (1500 ×)

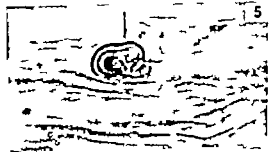
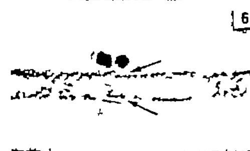
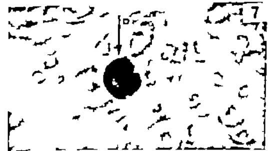
Fig 39 a, b and c. Longitudinal sections. Adult cat. I each of the pictures a nod of Ranvier is illustrated (arrows) A dense precipitate is present in the nod gap. (1500 ×)

Fig. 40 and b. Longitudinal sections. Ten day old kitten. Arrows point to nodes of Ranvier Not the precipitat in the node gap (1500 ×)


1

2

3a

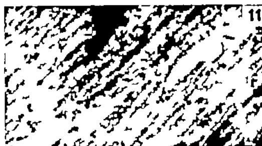
3b

4

5

6

7

8

9
B


10


11

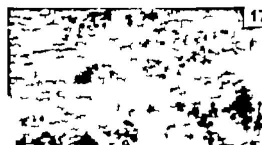

12a




13


14


15
B



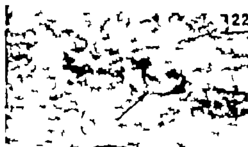

16

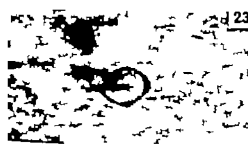
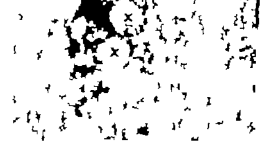



18

19

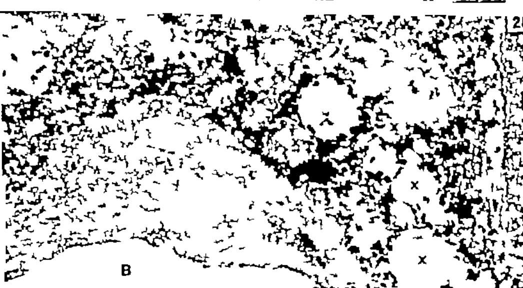
20

21

22

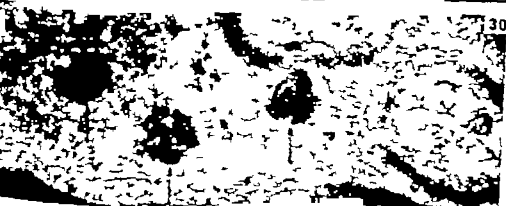
24

23


25



B
B

X
X
B

29







Kungl. Boktryckeriet P. A. Norstedt & Söner
Stockholm 1971
713070

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 363

STUDIES ON MECHANISMS CONTROLLING THE SECRETION OF NEUROTRANSMITTERS IN THE RABBIT HEART

BY
ÅKE WENNMALM

STOCKHOLM 1971

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 363

From the Department of Physiology Karolinska Institutet 104 01 Stockholm, Sweden

STUDIES ON MECHANISMS CONTROLLING THE SECRETION OF NEUROTRANSMITTERS IN THE RABBIT HEART

BY

ÅKE WENNMALM

STOCKHOLM 1971

Apart from certain unpublished results, the present thesis is based on the following papers

1 Preferential secretion of newly formed noradrenaline in the perfused rabbit heart. *Acta physiol. scand* 1970 *80* 428—429 (Together with L. Stjärne)

2 Quantitative evaluation of release and reuptake of adrenergic transmitter in the rabbit heart. *Acta physiol scand* 1971 (Preprint)

3 Prostaglandin E_1 as inhibitor of the sympathetic neuroeffector system in the rabbit heart *Life Sci* 1970, *9* I 931—937 (Together with P Hedqvist)

4 Comparison of the effects of prostaglandins E_1 E_2 and $F_{2\alpha}$ on the sympathetically stimulated rabbit heart. *Acta physiol, scand* 1971 (Preprint) (Together with P Hedqvist)

5 Inhibition by prostaglandin E_1 of parasympathetic neurotransmission in the rabbit heart. *Life Sci.* 1971 *10* I 465—470. (Together with P Hedqvist)

6. Inhibition of the release of adrenergic transmitter by a fatty acid in the perfusate from sympathetically stimulated rabbit heart. *Life Sci.* 1971 *10* I 471—479 (Together with L. Stjärne)

7 Increased nerve stimulation induced release of noradrenaline from the rabbit heart after inhibition of prostaglandin synthesis. *Acta physiol. scand* 1971 (Preprint) (Together with B Samuelsson)

These papers will be referred to by their arabic numerals.

ABBREVIATIONS USED

ACh = acetylcholine AChE = acetylcholine esterase CA = catecholamines DA = dopamine DMI = desmethylimipramine ETA = 5,8 11 14-eicosa-tetraynoic acid LU3-010 = 3,3-dimethyl-1 (methyl-aminopropyl) 1 phenyl pthalane NA = noradrenaline PBA = phenoxybenzamine PG = prosta glandin.

CONTENTS

†2—713086

A. Technique of heart perfusion

Rabbits of both sexes and mixed strains were used for the study. The weight of the animals varied from 1.1 kg to 2.4 kg. They were killed by a blow on the head and bled from the left carotid artery. The heart with intact left and right sympathetic supply was dissected out according to the method described by Huković and Muscholl (1962) and further developed by Löffelholz and Muscholl (1969 b). In some experiments the vagus nerves were kept intact and the sympathetic supply cut off. Following this, the heart was transferred to the perfusion apparatus. This consisted of two shanks, about 55 cm in height through which the perfusion solution passed down, while being continuously heated and aerated. The upper ends of the shanks were connected to reservoirs and their lower ends were joined together. A stop-cock made it possible to perfuse the heart from either of the two shanks and thus with solutions of different composition. The heart was perfused at a pressure of about 60 cm H_2O and a temperature of 37° C with Tyrode's solution of the following composition in mM: NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.6. The solution was aerated with 6.5 % CO_2 in O_2. Ascorbic acid, 20 μg/ml was added to the solution and usually also atropine (10^{-6} g/ml). A small cannula was inserted in a rubber tube between the perfusion apparatus and the heart to be used for infusion of drugs during the experiment. The drug solutions were of room temperature but the volume infused was always small enough not to affect the temperature of the perfusion medium passing into the heart.

The apex of the heart was connected to a strain gauge transducer. The longitudinal tension between base and apex and the frequency of the heart were recorded on a Grass Model 5D Polygraph. The tissue containing the fine postganglionic sympathetic nerve fibres or the vagal nerves were pulled through plastic tubes, with two circumferential platinum rings serving as electrodes. The electrodes were connected to separate Grass Model S5 stimulation units. The nerves were stimulated with trains of rectangular pulses 1 msec in duration, and of varying frequency and number of pulses. The voltage (usually 30—50 V) was adjusted to produce a current of 8 mA through the electrodes.

Perfusate from the heart was collected during nerve stimulation and for the following 90 sec, or until the inotropic response had vanished. Samples of the effluent from the heart were also taken between the nerve stimulations. The collected perfusate was immediately acidified and chilled.

B Fluorimetric analysis of NA

The NA content in the perfusate and the heart was determined with the trihydroxyindole method, as described by Euler and Lishajko (1961)

In experiments where the NA content of the heart was measured, the organ was immediately chilled and homogenized in an Ultra Turrax apparatus, in 0.4 M perchloric acid. After centrifugation at 9000×g for 15 min, the perchlorate in the supernatant was precipitated by titration with 4 M KOH to pH 4 The precipitated $KClO_4$ was removed by centrifugation at 9000×g for 10 min.

Sixty ml aliquots of the perfusate, or if the total volume was less, the entire perfusate, and the heart extracts were adjusted to pH 8.2—8.5 with NaOH after adding ethylenediamine-tetraacetic acid to a final concentration of 1 %. Catechols in the perfusate or heart extract were adsorbed on 0.5 g alumina (British Drug House) in a glass column of 9 mm inner diameter and after washing to pH 6 eluted with 6 ml 0.3 M acetic acid. One ml portions of the eluates were adjusted to pH 6.5 by adding 1 ml 0.5 M sodium acetate buffer (pH 6.5) ethylenediamine-tetraacetic acid 1 % and 0.4 ml 1 M NaOH. The samples were oxidized by adding 0.1 ml 0.25 % potassium ferricyanide for 3 min. The oxidation was stopped with 2 ml 0.2 % ascorbic acid in 4.4 M NaOH containing 2 % ethylene diamine added to stabilize the fluorescence (Euler and Lishajko 1961) The fluorescence was measured in an Aminco-Bowman photofluorimeter using standard filters (Scott 395 nm/Ilford Bright 490 nm) The mean recovery for NA added to the perfusate and carried through the procedure of purification was 76.6±1.4 % (mean±s.e.m., n = 27) The NA values are expressed as the base, not corrected for losses in the purification procedure.

Purification of NA in the perfusates or heart extracts was performed within 6 hrs after the experiment. Eluates were oxidized within 48 hrs after the purification.

C. Chromatographic separation of CA

Separation of DA and NA was performed by means of ion exchange chromatography according to Häggendal (1962) A strong cation exchange resin (Amberlite CG 120 type 2 mesh 200—400) was used in sodium form. Separation of NA and DA was performed on resin columns of 4×80 mm. A 4 ml aliquot of the alumina eluate was adsorbed on the column. Following this, the column was washed once with 3 ml of 1 M sodium acetate buffer (pH 6) and once with 3 ml redistilled water Elution of NA and DA was performed

using 1 M hydrochloric acid The eluate was separated into 1 6 ml fractions by an automatic fraction collector with drop counter (LKB Radi Rac) The NA in the separate fractions was determined fluorimetrically

D Analysis of labelled NA

For assay of the radioactivity of ^{14}C-NA and ^{3}H NA in perfusates, alumina eluates or chromatographic fractions, 1 ml aliquots were added to 10 ml Instagel (Packard Instr Inc.) or 0.5 ml aliquots to 6 ml absolute ethanol and 14 ml toluene containing 4 g/l of PPO and 0 1 g/l of POPOP The samples were counted in a Packard Tri-Carb or in an Intertechnique Abac SL 40 Liquid Scintillation Spectrometer for 10 or 20 min 2—3 times. In each series at least 3 blanks were measured. The radioactivity was expressed as cpm. Quenching was checked by internal standards.

E. Comment

In order to establish that the performed sympathetic nerve stimulation was postganglionic, and that the results described in the following chapters can thus be referred to events at the adrenergic nerve endings, 6 rabbits were given hexamethonium (2 mg/kg) 45 min before they were sacrificed Stimulation of the sympathetic nerves during 30 sec at a frecuency of 10/sec resulted in an outflow of 139±47 ng NA (mean±s.e.m., n = 6) A second nerve stimulation performed 15 min later resulted in an outflow of NA of 123±47 ng (n = 5) The corresponding NA outflow values in a control series, where no hex amethonium was given were 123±19 (n = 5) during the first and 105±17 (n = 5) ng NA during the second nerve stimulation, respectively Thus, there was no evidence that the method used to any significant extent involves stimulation of preganglionic fibres in the sympathetic nerves to the heart.

It is difficult to judge to what extent the level of NA in the effluent reflects the concentration of NA at the receptors. The correlation between these two parameters might be changed by variations in *e.g.* perfusion flow rate. How ever in the experiments presented in paper 2 there was no positive correla tion between the increase in outflow of NA and the increase in perfusion flow rate This observation makes it less probable that changes in the flow rate to any significant extent contributed to observed differences in the outflow of NA.

The validity and the reliability of the frequency recording method is complete, which is easily controlled by visual inspection

The reliability of the recording of the contractile force is probably also high as judged from the good agreement between the pattern of the inotropic response recorded during repetitive nerve stimulations. The validity of the recording of the contractile force is probably lower since the Langendorff method does not permit the heart to perform its normal work. For this reason, no detailed conclusions have been drawn from observations on the contractile force as recorded with the present method. However the advantage of the Langendorff preparation for the present purpose justifies its use, in spite of the low validity of the recording of the contractile force.

II TRANSMITTER ECONOMY REUPTAKE AND REUSE VERSUS *DE NOVO* SYNTHESIS

A. Introduction

Adrenergic nerve stimulation within the range which may be regarded as physiological does not result in reduction of the NA content of the tissue stimulated. The transmitter loss from the tissue is compensated for by an increased biosynthesis of the amine (Oliverio and Stjärne 1965) from tyrosine (Alousi and Weiner 1966, Gordon *et al.* 1966, Roth *et al.* 1967) and by reuptake of transmitter secreted (for a review *cf* Iversen 1967)

Estimates of the rate of synthesis of NA have frequently been based on studies of its turnover as reflected by the decline in the specific activity of NA after labelling the endogenous stores by administration of preformed ^{3}H NA or its precursors (Montanari *et al* 1963 Udenfriend and Zaltzman-Nirenberg 1963 Burack and Draskóczy 1964) Calculation of the NA synthesis rate by determining the conversion of ^{14}C-tyrosine to ^{14}C-NA (Sedvall *et al* 1968) indicated that the synthesis rate exceeds the turnover rate 2-3-fold. To explain this apparent discrepancy the authors suggested that the intraneuronal NA stores are divided into a small "secretory pool" with a rapid turnover and a larger "storage pool" with a slower turnover In order to study the fate of newly synthesized NA by a more direct method Kopin *et al.* (1968) analyzed the ratio ^{14}C-NA/NA in the effluent from the isolated perfused cat spleen during infusion of ^{14}C-tyrosine and simultaneous nerve stimulation They found that the specific activity of NA (^{14}C-NA/NA) in the perfusate increased during prolonged sympathetic nerve stimulation, and considerably exceeded the specific activity of NA in the organ immediately after the end of the stimulation period. This supports the concept of a small secretory pool of NA with a rapid turnover maintaining the secretion of NA in response to prolonged nerve stimulation. However these authors used a high frequency of nerve stimulation, which makes it difficult to evaluate the physiological significance of the results reported.

By contrast, Blakeley *et al.* (1969) reported that stimulation of the splenic nerves, in experiments with cat spleen perfused with blood containing PGE did not cause preferential secretion of newly formed NA. Moreover it was recently reported (Kupferman, Gillis and Roth 1970) that nerve stimulation at 5/sec in the superfused pulmonary artery preparation appeared to depress the biosynthesis of NA and that the proportion of newly synthesized NA leaving the organ was frequency-dependent it was 100 % in non-stimulated

arteries and 40 % in arteries stimulated at 25/sec. Thus, there is conflicting evidence concerning the importance of newly synthesized transmitter for the sustained secretion of NA during prolonged continuous nerve stimulation. The discrepancy between the results may to some extent be due to differences in species, tissues, preparations and techniques.

As shown by many authors, part of the NA secreted in response to sympathetic nerve stimulation is retained in the tissue, probably bound in the nerves themselves (for a review *cf* Iversen 1967) In order to determine the amount of NA secreted from the neurons, it is thus necessary to eliminate the trapping of NA in the tissue as completely as possible. Several techniques have been used for this purpose, such as high frequencies of nerve stimulation, high flow rates or drugs known to selectively block uptake of NA into the neurons. These different techniques for elimination of the tissue trapping of free transmitter increase the amount of NA leaving the stimulated organ to a variable extent, and thus lead to quantitatively different estimates of the extent of reuptake and reuse of transmitter

The drug most frequently used for inhibition of reuptake of NA is PBA, which however also blocks the α-receptors and thus the vasoconstriction in most preparations elicited by sympathetic nerve stimulation In the presence of PBA stimulation of the sympathetic nerves to the isolated cat spleen at frequencies of 10/sec or less causes a drastic rise in the outflow of NA from the organ, as compared to that seen without the drug On the other hand the enhancement of the nerve stimulation-induced outflow of NA by drugs such as cocaine or imipramine, which are regarded to be much more efficient than PBA as inhibitors of neuronal uptake of NA, have been inconsistent, varying from no effect to a moderate increase (*cf* Iversen 1967) Part of the explanation for this difference might be that PBA, but not cocaine, in addition to blocking neuronal uptake of NA also blocks the α-receptors and thus prevents vasoconstriction. However it cannot be excluded that some of the drugs used to block reuptake of the transmitter also affect the secretion of NA from the neurons.

The present chapter deals with studies of these problems in the rabbit heart The aim of the study was to find out to what extent the sustained secretion of NA on prolonged nerve stimulation is based on preferential secretion of newly formed NA as compared to reuptake and reuse of preformed transmitter

B Results (papers 1 and 2)

After a 10—15 min resting period in the perfusion apparatus, ^{14}C-labelled (New England Nuclear Corp uniformly labelled, spec. act 446 C/mole) and

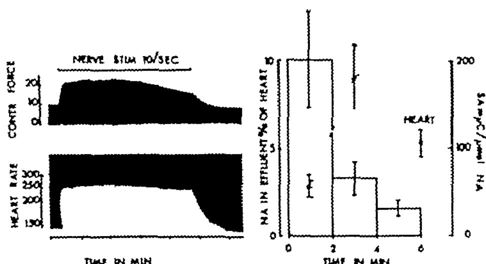


Fig. 1. Left: Inotropic and chronotropic response to prolonged supramaximal stimulation at 10/sec of the sympathetic nerves to the heart.
Right: outflow of NA (columns show mean ± s.e.m. during 3 consecutive 2-min periods of nerve stimulation, n = 7, n = 7, n =) and specific activity (dotted line: mean ± s.e.m., n = 7, n = 9, n = 1) in the perfusate. Specific activity of the heart at the end of the experiments (mean ± s.e.m., n = 8) marked at the 6 min point.
(from paper 1)

unlabelled tyrosine (2.5×10^{-7} to $? \times 10^{-2}$ M) was infused into the heart through a cannula immediately above the aorta. After 5 min of tyrosine infusion the sympathetic nerves to the heart were stimulated supramaximally at a frequency of 5 pulses/sec (4 expts) or 10 pulses/sec (9 expts) for 4 or 6 min. The perfusate was collected in 2 min periods. At the end of the experiment, the heart was rapidly homogenized. ^{14}C-NA in the effluent and in the heart was isolated by chromatography on alumina and Amberlite columns. The specific activity of NA (^{14}C-NA/NA) in the eluate fractions was estimated radiometrically and fluorimetrically.

During the nerve stimulation, the chronotropic response was maintained relatively constant, while the inotropic response, which was pronounced in the beginning of the nerve stimulation, usually decreased progressively (Fig. 1).

The effluent from the heart collected during the stimulation period contained both labelled and unlabelled NA. The total amount of transmitter overflowing decreased during continued nerve stimulation. In fact, the decrease in outflow of NA was more pronounced than the concomitant fading of the inotropic response. However, the specific activity of NA in the effluent gradually increased during the continued nerve stimulation: during the 2

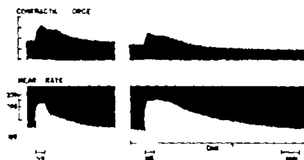


Fig. 2 Typical experiment showing th effect of sympathetic nerve stimulation (300 pulses at 10/sec) on the contractile force and frequency of the isolated rabbit heart. Left part shows control stimulation and right part shows stimulation in the presence of an uptake-blocking drug (DMI)
(from pape 2)

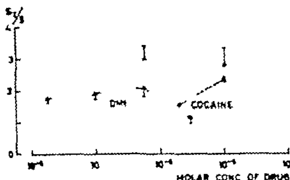
min period immediately preceding the termination of the stimulation it was 1.8 times that in the heart (P = 3 64** n = 8) at the end of the experiment (Fig 1) There seemed to be a slightly more pronounced rise in specific activity in hearts stimulated at 10/sec than in those stimulated at 5/sec. These findings are supported by recent experiments in this laboratory (Stjärne and Wennmalm to be publ.)

In the presence of drugs blocking uptake the mechanical response elicited by sympathetic nerve stimulation was dramatically changed. Thus, the chronotropic response was markedly prolonged (3.5 to 5.5 times) while its amplitude remained essentially unchanged (Fig 2) The prolongation of the chronotropic response to nerve stimulation caused by cocaine and desipramine but not by LU 3-010 was potentiated by α-blocking drugs. However the uptake blockers did not change the inotropic response to nerve stimulation, as recorded by the method used.

In the effluent from spontaneously beating hearts, no NA could be detected by the method used Stimulation of the sympathetic nerves for 30 sec at a frequency of 10 stimuli/sec caused an outflow of NA from the tissue of 221 ± 14 ng (mean ± s.e.m., n = 113) A second stimulation in ... ex periments performed 15 min after the first one ... distin (88 ± 3 % of first stimulation mean ± s.e.m. n ... of N

The uptake blocking drugs used did not increas spontaneously beating heart however they augr of NA in response to nerve stimulati (10^{-5} M) or desipramine (5.5×10^{-7} M duced outflow of NA by 133 ± 7 % (n ... 0 tively (Fig 3) However the outflow o at optimal concentrations of PBA (3

Fig 3 Ratio (mean ± s.e.m.) between outflow of NA during second (S_2, drug added) and first (S_1) nerve stimulation (300 pulses at 10/sec) Filled symbols show the effect of adding Hydergin (0.3 µg/ml) to the respecti drugs.
(from paper)



triptyline (10 M 250 ± 10 % n = 5) or LU 3-010 (10 M 199 ± 58 % n = 4) was significantly higher (Student s t test P = 2.8??** n = 22) than that in the presence of optimal concentrations of cocaine or desipramine (Fig 4) Hydergin (0.3 µg/ml) potentiated the effects of cocaine or desipramine on nerve stimulation induced outflow of NA to 180 ± 32 % (n = 7) and 187 ± 3 % (n = 5) respectively close to the range obtained for PBA, protriptyline and LU 3-010 (Fig. 3) In fact, there was no significant difference in the effect on the outflow of NA in response to nerve stimulation between PBA, protriptyline or LU 3-010 alone on the one hand, and cocaine or desipramine combined with Hydergin, on the other Neither Hydergin nor the β-blocking drug pronethalol potentiated the effect of LU 3-010 on the outflow of NA in response to nerve stimulation

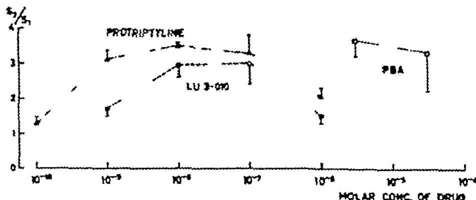


Fig. 4 Ratio (mean ± s.e.m.) between outflow of NA during second (S_2, drug added) and first (S_1) nerve stimulation (300 pulses t 10/sec)
(from paper 2)

The ability of the drugs to block the removal of circulating exogenous NA, at the drug concentrations found to be optimal with respect to potentiation of NA outflow on nerve stimulation, was studied at two different concentrations of NA in the medium, 1.7 μg/l and 25 μg/l. During perfusion with the lower NA concentration 85 ± 3 % (mean ± s.e.m., n = 8) and with the higher NA concentration 86 ± 2 % (n = 8) of the NA infused appeared in the effluent from the heart. Cocaine or desipramine (10^{-5} M and 5.5×10^{-7} M respectively) increased the amount of NA recovered in the effluent to 99 ± 3 % (n = 5). When PBA (3×10^{-6} M), protriptyline (10 M), LU 3-010 (10^{-7} M) or Hydergin (0.3 μg/ml) together with cocaine (10^{-5} M) or desipramine (5.5×10^{-7} M) were added, 99 ± 1 % (n = 11) of the NA appeared in the effluent. Thus, cocaine or desipramine on the one hand, and PBA, protriptyline, LU 3-010 or cocaine or desipramine together with Hydergin on the other *did not differ in their ability to block the uptake of exogenous NA.*

C. Concluding remarks

Sedvall *et al.* (1968) found that the values for NA synthesis rate calculated from determination of the ¹⁴C-NA found and the mean specific activity for tyrosine in the tissue, exceed earlier estimates, based on studies of NA turnover. They concluded that their findings support the concept that the transmitter is located in more than one pool intraneuronally (Trendelenburg 1961, Potter and Axelrod 1963, Chidsey and Harrison 1963, Sedvall and Thorson 1965) differing in function and with different turnover rates. The estimated specific activity of tyrosine in the study of Sedvall *et al.* was probably too high due to contamination of the isolated labelled tyrosine fraction with amphoteric radioactive material (Lewander and Jonsson 1968), their calculation of the NA synthesis rate was thus probably too low. Kopin *et al.* (1968) were the first to demonstrate, by a more direct method, that newly formed NA is secreted in preference to preformed NA, on prolonged nerve stimulation in the perfused cat spleen. Since inhibition of NA synthesis decreased the amount of transmitter leaving the organ in response to prolonged nerve stimulation, the authors suggested that mobilization of stored NA does not play as great a role in the maintenance of transmitter secretion as does *de novo* synthesis.

The present experiments (paper 1), where this concept was reexamined in the rabbit heart, clearly demonstrate that newly formed NA is secreted in *distinct preference in this organ, at a stimulation frequency which must be* considered physiological (Folkow 1952). The results are thus compatible with the view that the sustained secretion of transmitter in response to prolonged sympathetic nerve stimulation is dependent on preferential secretion of newly

formed NA and they show that this is true even at physiological nerve impulse frequencies.

The finding by *Kupferman, Gillis and Roth (1970)* in the sympathetically stimulated, superfused pulmonary artery that the proportion of preformed NA leaving the organ increased from 0 % during resting conditions to 60 % during stimulation at a frequency of 25 stimuli/sec, does not appear to be compatible with the concept of preferential secretion of newly synthetized NA. However considerable amounts of newly synthesized NA were lost into the bath even during resting conditions, probably as a result of spontaneous "leakage" of transmitter not associated with depolarization induced specific secretion. If this leakage is compensated for the figures given in this paper indicate a distinct preferential secretion of newly formed NA, both at 5 stimuli/sec and at 25 stimuli/sec.

Kopin *et al* (1968) reported that the specific activity for NA in the effluent from the spleen at the end of their experiments was more than eight times higher than the specific activity in the organ. In the present experiments with the rabbit heart the corresponding value was 1.8. The explanation for this difference is not quite clear One possibility is that the observed difference reflects true variations in the rate of synthesis of NA due to species and tissue differences. Another possible explanation would be that the adrenergic neurons of the cat spleen contain a relatively larger "storage pool, with a slow turnover than those of the rabbit heart where thus a greater proportion of the NA store would be located in the secretory pool, possibly in vesicles in "secretory position i.e. close to the axonal membrane This assumption is to some extent supported by the finding that the turnover of NA during resting conditions is much slower in the spleen than in the heart of the rat (Burack and Draskóczy 1964)

The marked prolongation of the chronotropic response to nerve stimulation in the presence of optimal concentrations of the different uptake blocking drugs is in good agreement with earlier reports in the literature (*Huković* 1959 Haefely Hürlimann and Thoenen 1964 Sigg Soffer and Gyermek 1963 Petersen *et al.* 1966) and supports the view (Rosell Kopin and Axelrod 1963) that a major proportion of the adrenergic transmitter secreted by nerve stimulation *is normally recaptured and that reuptake plays an important role* in the termination of action of the NA secreted from sympathetic nerves. The marked increase in the outflow of NA on nerve stimulation in the presence of uptake blocking drugs confirms earlier reports on this issue (Huković and Muscholl 1962, *Löffelholz and Muscholl 1970 a)* *The results are compatible* with the view that the maintained transmitter secretion on prolonged nerve stimulation is dependent on reuptake and reuse of liberated transmitter (Stjärne

ne 1964 Blakeley Brown and Geffen 1969) Similar conclusions were reached after experiments in intact rats (Bhagat and Friedman 1969) where accelerated synthesis of NA alone, in the presence of uptake blocking drugs did not compensate for the loss of amine from the heart in response to high nerve impulse frequencies. In this connection it is interesting, that perfusion of the isolated cat spleen with blood instead of a saline medium slightly reduces the overflow of transmitter in response to nerve stimulation. The significance of the reuptake mechanism may thus, if anything, be slightly underestimated when evaluated on the basis of results obtained in organs perfused with salt solutions.

The different uptake blockers used in the present experiments form two significantly separated groups, increasing the outflow of NA in response to nerve stimulation 2.? and 3.4-fold respectively compared to the preceding control stimulation. The significance of this difference will be discussed more in detail in chapter V However the drug experiments indicate that even at the very high flow rates used in the present study at least one half of the NA secreted in response to nerve stimulation is normally rebound.

The present data show that stimulation of the sympathetic nerves to the rabbit heart at physiological frequencies causes preferential secretion of newly formed NA. This suggests, that the sustained secretion of NA in response to prolonged nerve stimulation is dependent on *de novo* synthesis and preferential secretion of newly formed NA. However maintained transmitter secretion seems also to be dependent on reuptake and reuse of transmitter secreted from the nerves. The recapture mechanism thus serves the dual purpose of terminating the action of the transmitter secreted and maintaining secretion during prolonged periods of adrenergic nerve impulse activity

III EFFECTS OF EXOGENOUS PROSTAGLANDINS ON SECRETION OF AUTONOMIC NEUROTRANSMITTERS

A. Introduction

The morphology and function of adrenergic and cholinergic nerve endings have many features in common. The transmitter is stored in vesicles (Del Castillo and Katz 1955 Euler and Hillarp 1956) of about the same size It is probably secreted from these vesicles upon depolarization of the nerve terminal (Del Castillo and Katz 1957 *cf* Geffen and Livett 1971) The axoplasm contains particle-bound enzymes, MAO (Blaschko Hagen and Hagen 1957) and AChE (Whittaker 1959) respectively which degrade transmitter molecules outside the vesicles but inside the neuron. The secretion of transmitter upon depolarization is Ca^{++}-dependent in both types of neurons (Del Castillo and Stark 1952, Huković and Muscholl 1962, Vincenzi and West 1965)

The mechanisms involved in stimulation-secretion coupling in the two types of neurons are so far only known to a limited degree As mentioned, the presence of Ca^{++} is necessary in both adrenergic and cholinergic neurons Burn and Rand (1959) suggested that the impulse passing down the postganglionic sympathetic fiber releases ACh, which in its turn induces the secretion of NA In adrenergic nerves an enzymatic intermediary process has also been proposed to be involved (Kirpekar Prat and Yamamoto 1970) since low temperature and metabolic inhibitors decrease the nerve stimulation induced secretion of NA (Kirpekar Prat and Yamamoto 1970 Wennmalm, Hedqvist and Stjärne 1970) The spontaneous release of NA from the intraneuronal storage vesicles is also inhibited by low temperature (Euler and Lishajko 1963) and by metabolic inhibitors (Euler and Lishajko 1969)

The amount of transmitter secreted on nerve stimulation can also be altered by drugs. Thus the ganglionic blocking agent tetraethylammonium increases the amount of transmitter secreted from cholinergic (Koketsu 1958, Collier and Exley 1963) and adrenergic (Thoenen, Haefely and Staehelin 1967) neurons in response to nerve stimulation. The effect is probably due an increase in the duration of the local depolarization of the nerve terminals (Koketsu 1958, Thoenen, Haefely and Staehelin 1967)

Mechanisms involved in stimulation-secretion coupling seem to be the target of action for drugs like clonidin, and for biologically occurring ergones such as angiotensin, ACh and PG which have been shown to influence the nerve stimulation induced outflow of NA from sympathetically innervated tissues. Clonidin, 1.5×10^{-8} M, significantly inhibits the outflow of NA in response to

sympathetic nerve stimulation from the isolated rabbit heart at a concentration where its local anaesthetic effect is negligible (Starke and Schumann 1971) Angiotensin increases the secretion of NA from the isolated rabbit heart induced by nerve stimulation (Starke *et al.* 1970 Schümann, Starke and Werner 1970) In the same preparation Löffelholz and Muscoll (1969 a) found that ACh (10^{-9}—10^{-3} g/l) markedly inhibits the outflow of NA in response to nerve stimulation. The inhibitory effect observed with exogenous ACh can also be elicited by vagal nerve stimulation (Löffelholz and Muscholl 1970 b)

Prostaglandins of the E series also inhibit the outflow of adrenergic transmitter in response to nerve stimulation these studies have been performed in the cat spleen (Hedqvist and Brundin 1969 Hedqvist 1970 a) and to a limited extent in the isolated rabbit heart (Hedqvist and Wennmalm 1970 Hedqvist, Stjärne and Wennmalm 1970) Indirect evidence for an inhibiting action by PGE on the secretion of NA from adrenergic nerve terminals has also been found in the isolated vas deferens from various animals (Euler and Hedqvist 1969 Hedqvist and Euler 1971) In these studies, the action of PGE was probably mainly prejunctional since the effector response to exogenous NA was unchanged. It has also been shown in experiments on the cat spleen that PGE does not affect the reuptake of NA secreted in response to nerve stimulation (Hedqvist 1970 a) and that the inhibitory action of PGE_2 can be effectively counteracted by increasing the Ca concentration in the perfusion medium (Hedqvist 1970 c) On the basis of these results it was suggested that PG inhibits nerve stimulation induced secretion of NA by blocking the influx of Ca^{++} into the neurons induced by depolarization, or by preventing Ca^{++} from reaching specific reactive sites in the interior of the neuron, thus interfering with stimulation-secretion coupling.

The experiments reported in the present chapter were performed to further study the inhibitory effect of exogenous prostaglandins of the E series on the outflow of NA in response to sympathetic nerve stimulation in the rabbit heart, and to find out if the effect is specific for PG of the E series. The purpose was also to find out if prostaglandins exert a similar inhibitory effect on transmitter secretion from the cholinergic nerves of the heart.

B Results (papers 3 4, 5)

The marked chronotropic and inotropic responses to stimulation of the sympathetic nerves for 30 sec at a frequency of 10 pulses/sec ere found to be inhibited in a dose-dependent manner by PGE_1 and PGE_2. The two compounds were equally potent in inhibiting the chronotropic response to this type of stimulation (fig 5) The inhibition was weak at 3×10 M and became

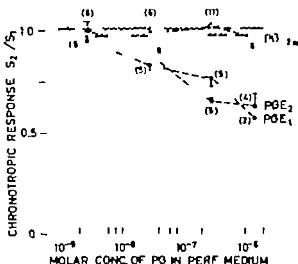


Fig 5. Effect of PGE_1 PGE_2 and $PGF_{2\alpha}$ on the chronotropic response to sympathetic nerve stimulation (10 pulses/sec, 30 sec) All values presented as ratio between 2nd and 1st stimulation. Hatched area control experiments in which PGs were omitted. Vertical bars: mean ± s.m. Figures within brackets number of experiments.
(from paper 4)

progressively more pronounced when the PG concentration in the medium was raised to 1.5×10^{-6} M, where the inhibition was about 40 %. The inotropic response as recorded with the method used, seemed to be more sensitive to PGE_1 than to PGE_2 (fig 6) Thus, at 3×10^{-8} M no depression of the inotropic response to nerve stimulation was seen in the presence of PGE_2 while PGE_1 at the same concentration reduced it markedly The more pronounced depression of the inotropic response caused by PGE_1 compared to PGE_2 was apparent throughout the dose range tested. $PGF_{2\alpha}$ did not influence the chronotropic or inotropic responses to sympathetic nerve stimulation at any of the dose levels

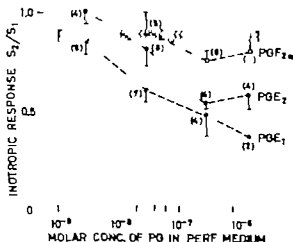


Fig. 6. Effect of PGE_1 PGE_2 and $PGF_{2\alpha}$ on the inotropic response to sympathetic nerve stimulation (10 pulses/sec, 30 sec) All values presented as ratio between 2nd and 1st stimulation. Hatched area: control experiments in which PG were omitted Vertical bars mean ± s.m. Figures within brackets number of experiments.
(from paper 4)

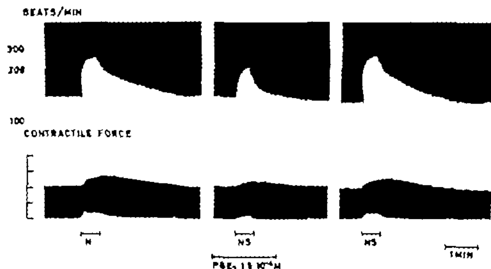


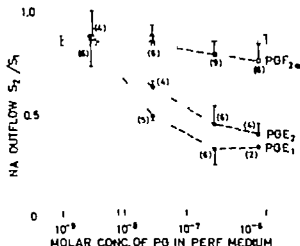
Fig 7 Chronotropic and inotropic responses to three periods of postganglionic sympathetic nerve stimulation (10 pulses/sec 30 sec) PGE_2 1.5×10^{-6} M infused during the second stimulation period.
(from paper 4)

tested (fig. 5 6) In some experiments a stimulation was given 15 min after the end of the infusion of prostaglandins. By that time the inhibition produced by the PGEs was no longer apparent and the response returned towards the preinfusion levels (fig. 7)

The outflow of NA in response to sympathetic nerve stimulation was also depressed in a dose-dependent manner by PGE_1 and PGE_2 (fig. 8) PGE_1 seemed to be slightly more effective than PGE_2 throughout the dose range tested at $3\times10^{-}$ M inhibiting the outflow by about 50 % (PGE_2 40 %) and at 1.5×10^{-6} M by about 70 % (PGE_2 60 %) $PGF_{2\alpha}$ did not inhibit the nerve stimulation induced outflow of NA at any of the dose levels tested

Infusion of NA (0.45 µg or 2.7 µg during 40 sec) caused an increase in heart rate and contractile force however the chronotropic response was weak on infusion of the lower dose of NA. A second NA infusion caused a chronotropic response of the same magnitude while the inotropic response was somewhat lower When PGE_1 (3×10^{-7} M to $3\times10^{-}$ M) or PGE_2 (1.5×10^{-6} M to 3×10^{-9} M) were infused prior to and during the second NA infusion no significant change in the chronotropic or inotropic responses to this type of stimulation was observed. The slight decrease in the chronotropic response to infusion of NA during simultaneous infusion of PGE_1 reported in paper 2 was thus not statistically significant.

Fig. 8. Effect of PGE_1, PGE_2 and $PGF_{2\alpha}$ on the outflow of NA in response to sympathetic nerve stimulation (10 pulses/sec, 30 sec). All values presented as ratio between 2nd and 1st stimulation. Hatched area: control experiments in which PGs were omitted. Vertical bars: mean ± s.e.m. Figures within brackets: number of experiments.
(from paper 4)



Stimulation of the left and right vagal nerve to the heart for 10 sec resulted in bradycardia, the degree of which was proportional to the stimulation frequency. On repeated stimulation there was no fading in the chronotropic response to vagal stimulation; the ratio between two repetitive stimulations was 1.08 ± 0.07 (mean ± s.e.m., n = 28). Infusion of PGE_1 (5×10^{-7} M to 8×10^{-7} M) markedly and reversibly counteracted the chronotropic response to vagal nerve stimulation (fig. 9). The inhibitory effect of PGE_1 did not appear to be correlated to the stimulation frequency. The average ratio between the second the the first stimulation in experiments where PGE_1 was infused during the second stimulation was 0.60 ± 0.03 (n = 12). This ratio is

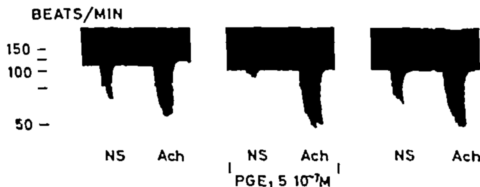


Fig. 9. Chronotropic responses to three parasympathetic nerve stimulations (1 pulse/sec, 10 sec) and infusions of acetylcholine. Second nerve stimulation and infusion of acetylcholine performed during infusion of PGE_1 (5×10^{-7} M).
(from paper 5)

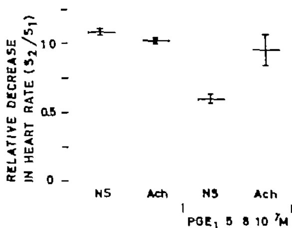


Fig 10 Ratio between chronotropic response to parasympathetic nerve stimulation (1—8 pulses/sec, 10 sec) or infusion of acetylcholine, during the second (S_2, PG infused when indicated) and during the first (S_1) stimulation or infusion.
(from paper 5)

statistically lower (P = 11 75*** n = 40) than that obtained in control experiments (1.08 ± 0 02) (fig 10)

Infusion of ACh (0 7 μg to 4.5 μg) also reduced the heart rate. The ratio between the negative chronotropic responses to two consecutive ACh infusions was 1 02 ± 0.02 (n = 9) Infusion of PGE did not change the chronotropic response of the heart to ACh (fig 9) the ratio between the response to the second and first ACh infusion in experiments, where PGF_1 was infused during the second ACh infusion, was 0.96 ± 0 11 (n = 4) (fig. 10)

C. Concluding remarks

The data presented clearly demonstrate that prostaglandins of the E series inhibit the autonomic neuroeffector system in the rabbit heart by a prejunctional action. These findings support the observations by Hedqvist (1970 b) and increase the probability that prostaglandins of the E series are capable of inhibiting transmitter secretion from sympathetic neurons in general independently of species and tissue. The present demonstration of a similar inhibitory effect in the parasympathetic nerves of the heart shows that the effect of PGE is not restricted to sympathetic nerves.

As mentioned earlier Hedqvist (1970 c) suggested that PGE inhibits NA secretion either by inhibiting Ca^{++} influx induced by depolarization or by preventing Ca^{++} from reaching specific reactive sites in the interior of the neuron The present results, which demonstrate a prejunctional inhibitory action of PGE_1 also in parasympathetic neurons, suggests a common mechanism of action of PGE in the two types of autonomic neurons. Since the secretion of ACh from cholinergic neurons requires Ca^{++} it seems likely that PGE may act on the Ca^{++} link in stimulation-secretion coupling

It has recently been shown (Sjöstrand 1971) that PGE_1 (3×10^{-8} M to 6×10^{-7} M) in a dose-dependent way depolarizes the isolated guinea pig vas deferens preparation, and in addition that the magnitude of evoked junction potentials are reduced by this compound. On the basis of these findings he suggested that the inhibitory effect of PGE on nerve stimulation induced outflow of NA might be explained by a similar depolarizing effect on the adrenergic nerve endings. The action potential in such hypopolarized nerve endings should result in a reduced influx of cations (including Ca^{++}). Thus electrophysiological evidence supports the concept that PGE may influence the secretion of autonomic transmitters by controlling the availability at critical sites within the nerve terminals, of Ca^{++}. If this is the level of action of PGE the finding of Hedqvist (1970 c) seems logical: increased Ca^{++} in the perfusion medium should overcome the inhibitory effect of exogenous PGE_2 on nerve stimulation induced outflow of NA.

The suggested hypothesis for the level of action of PGE might also explain why PGE fails to affect the stimulation-secretion coupling in the adrenal medulla (Miele 1969, Hedqvist and Stjärne 1971), which is also Ca^{++}-dependent (Douglas and Rubin 1961, 1963). In this organ, omission of sodium from the perfusion medium potentiates rather than depresses the CA secretion in response to ACh. ACh induces secretion of CA even in glands perfused with potassium phosphate Locke's solution, where the chromaffin cells might be assumed to be already depolarized (Douglas and Rubin 1963). Thus, secretion of CA from the adrenal medulla does not seem to require a propagated action potential in the chromaffin cell. In view of the hypothesis that PGE inhibits outflow of NA by hypopolarizing the cell membrane, it seems likely that such an inhibitory action might be less evident in cells which do not require a propagated action potential for secretion.

IV EVIDENCE FOR AN ENDOGENOUS INHIBITION OF ADRENERGIC TRANSMITTER SECRETION

A. Introduction

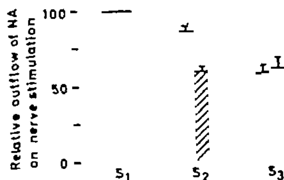
As shown in the previous chapter it is well established that exogenous prostaglandins inhibit the outflow of NA in response to sympathetic nerve stimulation in various tissues and animals by a prejunctional action (for references see chapter III) The inhibitory effect of PG seems to consist in depression of the secretion of NA from the sympathetic neurons.

Endogenous prostaglandins are present in many tissues and species (Bergström, Carlson and Weeks 1968) Their cellular and subcellular localization remains unclear and their physiological function is also unknown, although proposals have been made for their function at least in some organs (Euler 1936, Eliasson 1959 Horton and Main 1967 Brundin 1968)

PGE_2 has been shown to be released in response to nerve stimulation to the spleen of the dog (Davies, Horton and Withrington 1967 1968) and cat (Gilmore Vane and Wyllie 1968) In the dog spleen, the outflow of PGE_2 induced by nerve stimulation is inhibited by PBA. This drug has been shown to cause a marked potentiation of the nerve stimulation induced outflow of NA from the cat spleen (Brown and Gillespie 1957) the potentiation seems to be more marked than could be expected from its uptake blocking property alone (Malmfors 1965 *cf* Iversen 1967) In the same organ, antagonism between PGE_2 and PBA on the outflow of NA on nerve stimulation has been reported (Hedqvist 1969) From the latter findings it was suggested that PBA induced inhibition of an endogenous prostaglandin brake on transmitter secretion might be part of the reason for the striking difference between the effect of PBA on outflow of NA in response to nerve stimulation, and that of certain other drugs known to be even more efficient in blocking NA uptake, such as cocaine and DMI This suggestion is supported by the experiments in the rabbit heart presented in chapter II where cocaine and DMI in concentrations which completely blocked the uptake of exogenous NA did not cause as marked potentiation of the nerve stimulation induced outflow of NA as did PBA, protriptyline or LU 3-010

Reports in the literature from studies performed in different species and with different techniques thus seem to favour the concept of an endogenous prostaglandin-mediated braking mechanism, operating on the process of NA secretion in response to sympathetic nerve stimulation. However no definite

Fig 11 Outflow of labelled NA during three postganglionic stimulations of the sympathetic nerves (5 pulses/sec 15 or 30 sec) expressed as percent of the outflow of NA during the first nerve stimulation. Open columns heart perfused with rest perfusate Striped column: heart perfused with stimulation perfusate
(from paper 6)



proof for such a mechanism has hitherto been presented The experiments reported in this chapter were performed to find conclusive evidence for the postulated braking mechanism.

B. Results (papers 6, 7)

The aim of the experiments was to find out, if PG:s are released in response to sympathetic nerve stimulation from the rabbit heart, and in addition if they are released in amounts sufficient to depress the secretion of NA from the nerves of the heart. In order to prove that the secretion of sympathetic neurotransmitter is normally depressed by PG experiments were also carried out to study the effect of inhibition of the synthesis of endogenous PG in the heart, on nerve stimulation induced secretion of NA

When recipient hearts were perfused with RP (rest perfusate, collected from a spontaneously beating heart in a previous experiment) there was no difference between the chronotropic responses during three consecutive periods of sympathetic nerve stimulation. The nerve stimulation induced outflow of NA declined however somewhat, being 86 ± 4 % (mean ± s.e.m., n = 4) and 58 ± 4 % (n = 3) during the second and third stimulation period respectively relative to the first (fig 11)

Other hearts were perfused with RP during the first and third nerve stimulation and with SP (stimulation perfusate, collected from a sympathetically stimulated heart) during the second stimulation. The chronotropic response, which was 69 ± 3 beats/min (mean ± s.e.m., n = 20) during perfusion with RP was significantly (P = 3.53** n = 10, paired analysis) depressed to 59 ± 6 beats/min (n = 10) during perfusion with SP The outflow of NA in response to nerve stimulation was depressed to 60 ± 3 % (n = 8) when the

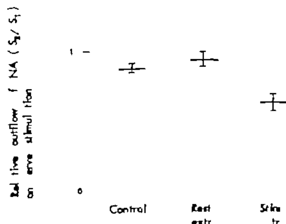


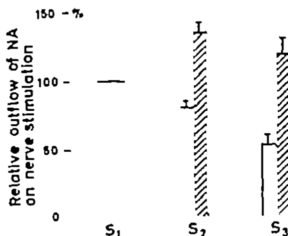
Fig 12 Ratio between outflow of NA during 2nd (S_2) and 1st (S_1) sympathetic nerve stimulation (300 pulses 10 pulses/sec) in control experiments and in experiments where the 2nd nerve stimulation was performed during infusion of acid lipid extract of rest perfusat (Rest.extr) and stimulation perfusate (Stim. extr) respectively
(from paper 6)

second stimulation was performed during perfusion with SP (fig 11) compared to that seen on perfusion with RP (86±4 %) The difference is statistically significant (P = 5 7** n = 12)

One SP was kept at pH 11 for one hour before it was adjusted to pH 8 0 and used for perfusion of a recipient heart. The chronotropic and NA outflow responses to nerve stimulation in this recipient heart (RP during the first and the third SP during the second stimulation) resembled the values obtained when hearts were perfused with RP during the entire experiment, indicating that the factor responsible for SP effects was inactivated at an alkaline reaction.

In order to purify and identify the active principle, SP was extracted for lipid soluble substances at alkaline (pH 8.5 or 11) and acid (pH 3) reaction. The extract (SE) dissolved in Tyrode's solution, was infused into a recipient heart and tested for capacity to inhibit the nerve stimulation induced outflow of NA. This capacity was estimated by determining the ratio outflow of NA during the second nerve stimulation (SE infused)/outflow of NA during the first nerve stimulation. As controls served the corresponding ratio obtained in hearts where RE (extract from rest perfusate) was infused during the second nerve stimulation. The ratio in the control experiments was 0.94±0 05 (mean±s.e.m., n = 6) When SP was extracted at alkaline reaction, the ratio between the outflow of NA during the second and the first nerve stimulation did not differ from the control ratio. However when SP was extracted at pH 3 the outflow of NA during the second nerve stimulation decreased compared to the first, giving a ratio of 0 63±0 06 (n = 6) (fig 12) This value is significantly lower (P = 4.03** n = 12) than that obtained in the

Fig. 13 Outflow of NA during three consecutive 2-min periods of postganglionic sympathetic nerve stimulation (5 pulses/sec) expressed as per cent of the outflow of NA during the first 2-min period. Striped columns: hearts infused with the prostaglandin synthesis blocker ETA during 3 min from the beginning of the second 2-min period.



control experiments, indicating that the factor responsible for the SP effects observed in the preceding series was extracted by the lipid solvent at an acid but not at an alkaline reaction.

Finally SE was prepared from stimulation perfusate from hearts previously infused with the prostaglandin synthesis inhibitor 5 8 11 14-eicosatetraynoic acid (ETA) When this SE was tested for capacity to inhibit the nerve stimulation induced outflow of NA as mentioned above, it was found that the ratio between the outflow values during the second and the first stimulation resembled that obtained when RE was infused prior to the second stimulation. Thus, it appeared, that the prostaglandin synthesis blocker ETA inhibited the outflow from the nerve stimulated heart of the substance inhibiting NA outflow

In another series of experiments, the sympathetic nerves were continuously stimulated for 6 min at a frequency of 5/sec, and the effl ent was collected during two min periods. The outflow of NA, which was 116 ± 31 ng (mean $\pm$ s.e.m., n = 10) during the first two min, declined during the con tinued stimulation, to 80 ± 5 % (mean $\pm$ s.e.m., n = 5) and 53 ± 7 % (n = 4) during the second and third two min periods, respectively (fig 13) When ETA was infused during three min from the beginning of the second two min period, the outflow of NA increased to 135 ± 7 % (n = 5) during the second and 119 ± 11 % (n = 4) during the third two min periods of nerve stimula tion. The difference in outflow of NA during the second and third period relative to the first, between ETA and control experiments is statistically signifi cant (P = 6 67*** n = 10 and P = 5.26** n = 8 respectively)

When NA, 25 μg/l, was added to Tyrode's solution, 89 ± 4 % (mean $\pm$ s.e.m.,

n = 3) of the amine appeared in the perfusate. When ETA was infused prior to perfusion with NA-containing Tyrode's solution 82 ± 6 % (n = 3) of the amine appeared in the effluent. Thus, there was no evidence that ETA blocks the uptake of exogenous NA.

In order to find conclusive evidence that the inhibiting substance released from the heart in response to sympathetic nerve stimulation belongs to the family of prostaglandins, the following experiment was performed. SE prepared from 800 ml stimulation perfusate was purified by silicic acid chromatography. Half of the eluate was divided in four fractions, which were incubated at 37° C for two hours with 15-hydroxyprostaglandin dehydrogenase and NAD⁺, 15-hydroxyprostaglandin dehydrogenase only, NAD⁺ only and without enzymes, respectively (Änggård and Jonsson 1971). The fractions were tested for smooth muscle stimulating activity on an isolated rat stomach. The fraction incubated with both enzymes was found biologically inactive, while the remaining three fractions all possessed smooth muscle stimulating activity. Since 15-hydroxyprostaglandin dehydrogenase is specific for the 15-hydroxyl group in the compound and since the resulting product, 15-keto-prostaglandin is biologically inactive, the difference in activity between the incubated samples demonstrates the presence of PG in the effluent from the stimulated rabbit heart.

The remaining half of the silicic acid eluate was subjected to thin layer chromatography in system AII (Gréen and Samuelsson 1964). The areas corresponding to PGE_1 and PGE_2 were scraped off and extracted in ether. After evaporation the residue was dissolved in Tyrode's solution and tested on the rat stomach. Both extracts were found biologically active indicating the presence of both PGE_1 and PGE_2 in the effluent from the sympathetically stimulated heart.

C. Concluding remarks

Perfusion of recipient hearts with SP but not with RP significantly reduced the chronotropic and NA overflow responses to sympathetic nerve stimulation. This demonstrates the presence of material in SP which inhibits the outflow of NA in response to nerve stimulation. This material is not NA, since addition of the amine to RP at relevant concentrations did not affect the outflow of NA on nerve stimulation.

In order to classify the chemical properties of the inhibiting material, lipids were extracted from SP and the extract was tested for capacity to inhibit nerve stimulation induced outflow of NA. Such a capacity was observed when the lipid extraction was performed at pH 3, but not at pH 8.5 or 11. These

findings classify the active material as fatty acid(s). Extracts from RP possessed no inhibitory activity.

When SP was kept at pH 11 for one hour before lipid extraction at pH 3 no inhibitory effect on the nerve stimulation induced outflow of NA was observed, indicating destruction of the inhibitory material at an alkaline reaction.

The finding that the material capable of inhibiting the outflow of NA on nerve stimulation had the character of a fatty acid and was labile at alkaline reaction strongly suggested that it belongs to the prostaglandin family. In order to demonstrate the presence of PGs in SE, some extracts were tested for smooth muscle stimulating activity on an isolated rat stomach. Both RE and SE were biologically active in this respect, however, the activity was always higher in SE. SE was also subjected to further purification on silicic acid followed by incubation with PG-catabolizing enzymes or separation on thin layer chromatography. The results clearly demonstrated the presence in SE, and thus in SP, of PGE_1 and PGE_2. From these findings, it seems extremely likely that endogenous PGEs, released in response to sympathetic nerve stimulation are responsible for the inhibitory action of SP on nerve stimulation induced outflow of NA.

Since it has been shown that ACh in the perfusion medium inhibits the secretion of NA from the rabbit heart in response to sympathetic nerve stimulation (Löffelholz and Muscholl 1969 a), it might be argued, that the inhibitory action of SP could be due to the presence of ACh in SP released from parasympathetic neurons accidentally included in the sympathetic nerve stimulation. However, even the minimum concentration of ACh required to affect NA secretion, 1 ng/ml, is unlikely to result from accidental vagal stimulation. Furthermore, most recipient hearts were given atropine (10^{-6} g/ml), which abolishes the inhibitory action of ACh; there does not seem to be any difference in outflow inhibiting capacity by SP on hearts given atropine compared to those where the drug was omitted. The inhibitory material in SP was extractable in ethyl acetate at an acid reaction, this applies to ACh only to a limited extent. Finally, the inhibitory material in SP was not released when prostaglandin synthesis was blocked (below). Taking these facts into consideration, it seems most unlikely that ACh to any significant extent contributed to the inhibitory action of SP on the nerve stimulation induced outflow of NA.

When the endogenous synthesis of PGs was inhibited by ETA, infused into the perfusion medium, the outflow of NA from the organ in response to sympathetic nerve stimulation was increased. The enhanced outflow of NA does not seem to be due to impaired reuptake of transmitter secreted, since ETA

did not affect uptake of exogenous NA in the heart. Neither can the increased outflow of NA caused by ETA be explained in terms of a diminished enzymatic inactivation of the transmitter since the NA secreted in response to nerve stimulation in this preparation is enzymatically degraded to less than 10 % (paper 6) Thus it seems most likely that ETA treatment augments the secretion of transmitter in response to depolarization

The finding that inhibition of the local formation of prostaglandins is accompanied by an increased secretion of transmitter per nerve impulse seems to provide conclusive evidence for the existence of a prostaglandin-mediated control mechanism normally operating on the neurons to limit secretion of the sympathetic transmitter This supports the interpretation of the data found during experiments in the rabbit heart presented in Chapter II Although these were performed with a difference technique and a different approach the results indicated the existence of a mechanism limiting transmitter secretion. It was found (paper 2) that different uptake blocking drugs at concentrations sufficient to completely inhibit removal of exogenous NA from the perfusion medium, increased the nerve stimulation induced outflow of NA to a different extent. Thus cocaine and DMI increased the outflow by about 100 % while PBA protriptyline and LU 3-010 increased the outflow by more than 200 % It is likely that PBA, protriptyline and LU 3-010 in addition to blocking the reuptake of transmitter liberated augment the secretion of NA per nerve impulse they might do this by inhibiting the prostaglandin-mediated "braking" mechanism.

V GENERAL DISCUSSION

The aim of the present study was firstly to analyze the relative importance of synthesis and reuptake of NA for the sustained transmitter secretion in response to prolonged stimulation of the sympathetic nerves of the rabbit heart. Secondly, the purpose was to seek conclusive evidence for the earlier suggested hypothesis that the secretion of sympathetic transmitter is normally limited by a prostaglandin mediated "braking" mechanism. Finally, the intention was to find out if exogenous prostaglandins exert the same inhibitory effects on transmitter secretion in the parasympathetic neurons of the rabbit heart.

The concept of preferential secretion of newly formed NA is at present under debate. Sedvall *et al.* (1968) found that the NA synthesis rate, as determined by the conversion of ^{14}C-tyrosine to ^{14}C-NA exceeds earlier estimates, based on the rate of disappearance of labelled NA, by a factor of 2—3. They suggested that the intraneuronal NA store is divided into a small "secretory" pool with a rapid turnover and a larger "storage" pool with a slower turnover. However, since the labelled tyrosine was not separated from contaminating amphoteric material (*cf.* Lewander and Jonsson 1968) the NA synthesis rate may have been underestimated.

The issue of possible preferential secretion of newly synthesized transmitter has also been studied in isolated perfused organs. By comparison of the specific activity (^{14}C-NA/NA) of transmitter synthesized from ^{14}C-tyrosine in the effluent from the isolated perfused spleen, Kopin *et al.* (1968) found that newly synthesized NA is preferentially secreted from this organ. They also reported that blocking the NA synthesis with α-methyltyrosine during continuous nerve stimulation reduced the amount of transmitter secreted. Their conclusion was that preferential secretion of newly synthesized transmitter is important for the sustained secretion of transmitter during prolonged nerve stimulation. By a similar method, Besson *et al.* (1969) found that newly synthesized DA is secreted preferentially from slices of rat striatum. However, Blakeley *et al.* (1969) perfusing the isolated cat spleen with blood containing PGE$_1$ found no evidence for preferential secretion of newly synthesized transmitter in response to repeated trains of 1000 stimuli delivered at 10/sec. Similar results have been reported after experiments in the superfused pulmonary artery of rabbits (Kupferman, Gillis and Roth 1970). In the latter study, however, if unspecific leakage is accounted for, recalculation on the basis of the results reported in fact indicates that newly synthesized transmitter was preferentially secreted (*cf.* chapter II). Costa (1970) found the evidence presented by Kopin

et al. and by Besson *et al.* insufficient to prove that newly synthesized transmitter is preferentially secreted and presented results of his own, indicating that the transmitter secreted from the rat brain has the same specific activity as that retained in the organ.

In the present study this issue was reexamined in the isolated rabbit heart, using physiological stimulation frequencies. In every experiment presented in paper 1 and in a recently performed experimental series continued stimulation of the sympathetic nerves to the heart during simultaneous infusion of ^{14}C-tyrosine resulted in a progressively increasing specific activity of NA in the effluent from the organ at the end of the experiment the specific activity in the effluent was consistently higher than that in the heart. The experiments thus clearly demonstrate that newly synthesized NA is preferentially secreted from the heart during prolonged periods of sympathetic nerve stimulation at physiological frequencies they support the hypothesis that preferential secretion of newly formed NA may be even more important for the sustained secretion *of transmitter than is mobilization of stored NA.*

However the sustained secretion of NA during prolonged periods of adrenergic activity seems to require not only *de novo* synthesis but also an intact reuptake mechanism. Initially the recapture mechanism was mainly interpreted as a means for termination of action of the transmitter secreted (Rosell Kopin and Axelrod 1963) This view was based on observations in several tissues and species (*cf* Iversen 1967) that drugs known to block neuronal uptake of NA potentiated and prolonged the mechanical response to sympathetic nerve stimulation In view of the potency of the recapture mechanism it has also been suggested that reuptake and reuse of transmitter secreted might facilitate transmitter economy (Stjärne 1964) This hypothesis has recently been supported (Blakeley Brown and Geffen 1969) by experiments in the cat spleen, where prolonged nerve stimulation after treatment with drugs blocking the uptake mechanism leads to depletion of the transmitter stores, and to a markedly diminished secretion of NA in response to repetitive nerve stimulations The results presented in paper 2 further support this view The uptake blocking drugs used increased the nerve stimulation induced outflow of NA 2.2 to 3.4 times. The uptake mechanism was thus capable to rebind at least one half of the amount of NA secreted even at the high rates of flow through the perfused heart. In addition to terminating the action of the transmitter secreted, such an efficient reuptake mechanism should also by allowing reuse of transmitter be of importance for transmitter economy

The first observation which raised the possibility that exogenous PG:s may decrease the outflow of NA in response to sympathetic nerve stimulation was made by Brundin (unpubl obs) who found that the influence by PG:s on the

mechanical response to hypogastric nerve stimulation of the rabbit oviduct hardly could be explained by a postjunctional effect only This hypothesis was supported by the finding that exogenous PGE_1 decreases the secretion of NA in response to sympathetic nerve stimulation in the cat spleen (Hedqvist and Brundin 1969) After that, Hedqvist (1970 b) has presented convincing evidence that exogenous PGEs exert an inhibitory effect on the secretion of NA in response to sympathetic nerve stimulation on adrenergic neurons in several tissues and species. The finding that a similar inhibitory effect can be elicited even in parasympathetic neurons (paper 5) makes it probable that prostaglandins are capable of inhibiting the secretion of transmitters from post ganglionic autonomic neurons in general.

On the basis of the observations on inhibitory effects of exogenous PGs on the secretion of sympathetic neurotransmitter Hedqvist (1969 1970 b) introduced the hypothesis that endogenous PGs may play a role in a feed back mechanism controlling transmitter secretion from adrenergic neurons. There is some evidence in the literature which supports such a hypothesis. PGs are known to be released in response to sympathetic nerve stimulation from the dog and cat spleen (Davies, Horton and Withrington 1967 1968, Gilmore Vane and Wyllie 1968) PBA inhibits this release (Davies, Horton and Withrington 1967) and in addition causes a large increase in the amount of transmitter overflowing from the spleen in response to nerve stimulation The enhancing effect of PBA on transmitter overflow from the spleen is counteracted by PGE_2 (Hedqvist 1969) This indicates that part of the potentiating effect of PBA on outflow of NA during nerve stimulation may be due to removal of an endogenous prostaglandin-mediated "braking mechanism (Hedqvist 1969)

The experiments reported in papers 6 and 7 provide conclusive evidence for the existence of such an endogenous prostaglandin-mediated "braking" mechanism. It is shown that PGE_1 and PGE are released from the rabbit heart in response to sympathetic nerve stimulation, apparently in amounts capable to inhibit the secretion of NA from the neurons. It is also shown that the release of PGEs is much smaller d ring resting conditions. Finally infusion of the PG synthesis blocker ETA inhibits the nerve stimulation induced outflow of PGE from the heart and augments the secretion of NA.

This proves that transmitter secretion from the adrenergic neurons of the rabbit heart is normally limited by n endogenous, prostaglandin-mediated mechanism. There is at present no information concerning the existence of a similar mechanism operating on parasympathetic neurons.

The present experiments in the rabb t heart make it likely that endogenous "braking mechanisms may control transmitter secretion in other adrenergic neurons as well and possibly also in cholinergic neurons. As to the biological

usefulness of such a mechanism the field is open to speculation. One possibility is that a uniform increase in impulse frequency throughout the sympathetic nervous system might result in differentiation of the motor responses due to the presence or absence of PGs in various organs and organ systems, leading to unequal degrees of restriction of transmitter secretion. Another possibility is suggested by the fact that the sympathetic neurons discharge within relatively narrow frequency limits. If one assumes that the endogenous inhibition of transmitter secretion is unequally efficient at different impulse frequencies, the spectrum of possible motor responses would by widened.

VI SUMMARY

In the present study of mechanisms controlling the secretion of neurotransmitters in the rabbit heart the major interest was focused on the secretion of adrenergic transmitter in response to sympathetic nerve stimulation. The most important findings were the following

1 Continued sympathetic nerve stimulation at physiological frequencies causes preferential secretion of newly formed transmitter from the adrenergic neurons. This finding is consistent with the view that preferential secretion of newly synthesized NA is necessary for the sustained transmitter secretion.

2. At physiological stimulation frequencies, at least one half of the transmitter secreted is rebound in the tissue. This finding supports the concept that reuptake of NA secreted in response to depolarization is necessary for a rapid termination of action of the transmitter secreted In addition it is consistent with the view that transmitter economy is to a large extent based on reuptake and reuse of NA.

3 Exogenous PGE$_1$ and PGE$_2$ depress the outflow of NA from the heart in response to sympathetic nerve stimulation. The inhibitory effect on the secretion of NA from the adrenergic neurons is dose-dependent and reversible.

4 Exogenous PGE$_1$ reversibly inhibits the negative chronotropic response to parasympathetic nerve stimulation. Since the response to infusion of ACh is unaffected by PGE$_1$ it is concluded that the observed effect of PGE$_1$ is prejunctional.

5 The secretion of NA induced by adrenergic nerve stimulation appears to be normally restricted by an endogenous prostaglandin-mediated "braking" mechanism PGE$_1$ and PGE$_2$ could be isolated and identified in the effluent from the stimulated heart. Inhibition of the synthesis of prostaglandins removed the "braking" mechanism and led to an increased secretion of NA in response to nerve stimulation.

VII REFERENCES

Aloud, A. and N Weiner The regulation of norepinephrine synthesis in sympathetic nerves Effect of nerve stimulation, cocaine, and catecholamine-releasing agents. *Proc nat. Acad. Sci.* (*Wash.*) 1966. *56* 1491—1496

Änggård, E. and C. E. Jonsson, Efflux of prostaglandins in lymph from scalded tissue. *Act physiol. scand* 1971 (in press)

Bergström, S. L. A. Carlson and J R. Weeks, The prostaglandins A family of biologically active lipids. *Pharmacol. Rev* 1968 *20* 1—48.

Besson, M. J A. Cheramy P Feltz and J Glowinski, Release of newly synthetized dopamine from dopamine-containing terminals in the striatum of the rat. *Proc nat. Acad. Sci.* (*Wash.*) 1969 *62* 741—748.

Bhagat, B. and E. Friedman, Factors involved in maintenance of cardiac catecholamine content relative importance of synthesis and re-uptake. *Brit J Pharmacol.* 1969 *37* 24—33

Blakeley A. G H., G L. Brown, D P Dearnaley and V Harrison, The effect of nerve stimulation on the synthesis of ^{3}H noradrenaline from ^{3}H tyrosine in the isolated blood-perfused cat spleen. *J Physiol.* (*Lond*) 1969 *200* 59—60P

Blakeley A. G H., G L. Brown and L. B. Geffen, Uptake and reuse of sympathetic transmitter in the cat spleen. *Proc roy Soc B* 1969 *174* 51—68.

Blaschko H., J M. Hagen and P Hagen, Mitochondrial enzymes and chromaffin granules. *J Physiol.* (*Lond*) 1957 *139* 316—322.

Brown, G L. and J S. Gillespie, The output of sympathetic transmitter from the spleen of the cat. *J Physiol.* (*Lond.*) 1957 *138* 81—102

Brundin, J The effect of prostaglandin E_1 on the response of the rabbit oviduct to hypogastric nerve stimulation. *Act physiol. scand* 1968 *73* 54—57

Burack, W R. and P R. Draskóczy The turnover of endogenously labelled catecholamine in several regions of the sympathetic nervous system. *J Pharmacol. exp Ther* 1964 *144* 66—75

Burn, J H. and M. J Rand, Sympathetic postganglionic mechanism. *Nat* (*Lond.*) 1959 *184* 163—165

Chidsey C. A. and D C. Harrison, Studies on the distribution of exogenous norepinephrine in the sympathetic neurotransmitter store. *J Pharmacol. exp Ther* 1963 *140* 217—225

Collier B. and K. A. Exley Mechanism of the antagonism by tetraethylammonium of neuromuscular block due to *d*-tubocurarine or calcium deficiency *Natur* (*Lond.*) 1963 *199* 702—703

Costa, E., Simple neuronal models to estimate turnover rate of noradrenergic transmitters *in vivo* In "*Advances in biochemical psychopharmacology*" vol. 2. Eds. E Costa and E. Giacobini. Raven Press. 1970.

Davies, B. N E. W Horton and P G Withrington, The occurrence of prostaglandin E in splenic venous blood of the dog following splenic nerve stimulation. *J Physiol* (*Lond.*) 1967 *188* 38—39P

Davies, B. N E. W Horton and P G Withrington, The occurrence of prostaglandin E_2 in splenic venous blood of the dog following splenic nerve stimulation *Brit J Pharmacol.* 1968 *32* 127—135

Del Castillo, J and B. Katz, Local activity at a depolarized nerve-muscle junction. *J Physiol. (Lond.)* 1955. *128* 396—411
Del Castillo J and B. Katz, La base quantale de la transmission neuromusculaire In "*Microphysiologie comparé d lements excitables* Coll. internat. C.N.R.S. Paris. 1957 *67* 245—258
Del Castillo J and L. Stark, The effect of calcium ions on the motor end-plate potentials. *J Physiol. (Lond.)* 1952. *116* 507—515
Douglas, W W and R. P Rubin, The role of calcium in the secretory response of the adrenal medulla to acetylcholine *J Physiol. (Lond.)* 1961 *159* 40—57
Douglas, W W and R. P Rubin, The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. *J Physiol. (Lond.)* 1963 *167* 288—310.
Eliasson R., Studies on prostaglandin occurrence formation and biological actions. *A ta physiol. scand* 1959 *46* Suppl. 158, 1—73
Euler U S. On the specific vaso-dilating and plain muscle timulating substances from accessory genital glands in man and certain animals (prostaglandin and vesiglandin) *J Physiol. (Lond.)* 1936. *88* 213—234
Euler U S. v and P Hedqvist, Inhibitory action of prostaglandins E and E on the neuromuscular transmission in the guinea pig vas deferens. *A ta physiol. scand* 1969. *77* 510—51..
Euler U S v and N.-A. Hillarp E idence for the presence of noradrenaline in submicroscopic structures of adrenergic axons. *Natu (Lond.)* 1956. *177* 44—45
Euler U S v and F Lishajko Improved technique for the fluorimetric estimation of catecholamines. *Act physiol. scand* 1961 *51* 348—355
Euler U S v and F Lishajko, Catecholamine release and uptake in isolated adrenergic nerv granules. *A ta physiol scand.* 1963 *57* 468—480.
Euler U S and F Lishajko Effects of some metabolic co-factors and inhibitors on transmitter release and uptake in isolated adrenergic nerv granules. *Act physiol. scand* 1969 *77* 298—307
Folkow B., Impulse frequency in sympathetic vasomotor fibres correlated to the release and elimination of the transmitter *A ta physiol. scand* 1952. *25* 49—76.
Folkow B J Häggendal and B. Lisander Extent of release and elimination of noradrenaline at peripheral adrenergic nerve terminals. *Act physiol. scand* 1967 *72.* Suppl. 307 1—38
Geffen, L. B. and B G Livett, Synaptic vesicles in sympathetic neurons. *Physiol. Rev* 1971 *51* 98—157
Gilmore, N J R. Vane and J H. Wyllie Prostaglandins released by the spleen. *Natu (Lond.)* 1968. *218* 1135—1140.
Gordon, R., J V O Reid, A. Sjoerdsma and S Udenfriend Increased synthesis of norepinephrine in the rat heart on electrical stimulation of th stellate ganglia. *Mol. Pharmacol.* 1966 2 610—613
Green, K. and B. Samuelsson, P ostaglandins and elated factors XIX. Thin-layer chromatography of prostaglandins. *J Lipid Res.* 1964 *5* 117—120.
Haefely W A. Hürlimann and H Thoenen, A quantitative study of the effect of cocaine on the response of the cat nictitating membrane to nerve stimulation and to injected noradrenaline. *Brit J Pharmacol.* 1964 *22* 5—21
Häggendal, J On the use of strong exchange resins for determinations of small amounts of catecholamines *S and J lin Lab I vest* 196.. *14* 537—544

Hedqvist, P Antagonism between prostaglandin E_2 and phenoxybenzamine on nor adrenaline release from the cat spleen. *Acta physiol. scand* 1969 *76* 383—384

Hedqvist, P Control by prostaglandin E_2 of sympathetic neurotransmission in the spleen. *Lif Sci.* 1970 a. *9* I 269—278.

Hedqvist, P Studies on the effect of prostaglandins E_1 and F on the sympatheti neuromuscular transmission in some animal tissues. *Act physiol. sca d* 1970 b. *79* Suppl. 345 1—40

Hedqvist, P Antagonism by calcium of the inhibitory action of prostaglandin E_2 on sympathetic neurotransmission in the cat spleen. *Acta physiol. scand* 1970 c. *80* 269—275

Hedqvist, P and J Brundin, Inhibition by prostaglandin E of noradrenaline release and of effector response to nerve timulation in the cat spleen. *Lif Sci.* 1969 *8* I. 389—395

Hedqvist, P and U S. v Euler Prostaglandin-induced neurotransmission failure in the field-stimulated isolated vas deferens. *Neuropharmacology* 1971 (S bmitted for publication)

Hedqvist, P and L. Stjärne, Effect of prostaglandin E_2 on the transmitter release from sympathetic nerves and on hormone secretion from the adrenal medulla. 1971 (To be publ.)

Hedqvist, P L. Stjärne and Å. Wennmalm, Inhibition by prostaglandin E_2 of sympa thetic neurotransmission in the rabbit heart. *A t physiol. scand.* 1970 *79* 139—141

Hedqvist, P and Å. Wennmalm, Inhibition by prostaglandin E of the sympatheti neuromuscula transmission in the rabbit heart. *Act physiol. scand* 1970. *79* 19A—20A.

Horton E W and I H M. Main, Identification of prostaglandins in central nervous tissues of the cat and chicken. *Brit. J Pharmacol.* 1967 *30* 582—602.

Huković, S., Isolated rabbit atria with sympathetic nerve supply *Brit J Pharmacol.* 1959 *14* 372 -376

Huković, S. and E. Muscholl, Die Noradrenalin-Abgabe aus dem isolierten Kaninchen-herzen bei sympathischer Nervenreizung und ihre pharmakologische Beeinflussung. *Naunyn-S hmied berg' A h. f Path. Pharmak.* 1962. *244* 81—96.

Iversen, L. L., The uptake and storage of noradrenaline in sympathetic nerves. *Cam bridg Univ P ess* 1967

Kirpekar S M., J C. Prat and H. Yamamoto, Effects of metabolic inhibitors on nor epinephrine release from the perfused spleen of the cat. *J Pharm col. Ther* 1970 *172* 34 —350

Koketsu, K Action of tetraethylammon um chloride on neuromuscula transmission in frogs. *Amer J Physiol.* 1958. *193* 213— 18

Kopin, L J G R. Breese, K. R. Krauss and V K. Weise, Selective release of newly synthesized norepinephrine from th cat spleen during sympathetic nerve stimulation *J Pharmacol. Ther* 1968. *161* 271—278.

Kupferman, A., C. N Gillis and R. H. Roth, Influence of sympathetic nerve stimulation on conversion of H^3-tyrosine to H^3-catecholamine and on H^3-norepinephrine dispo sition in rabbit pulmonary artery *J Pharma ol. Ther* 1970 *171* 214—222

Lewander T and J Jonsson, Isolation and determination of free endogenous and radio-active tyrosine in tudies on catecholamine synthesis in the rat brain. *Lif Sci.* 1968. 7 II 387—394

Löffelholz, K. and E. Muscholl, Die Hemmung der Noradrenalin-Abgabe durch Acetylcholin am sympathisch gereizten, isolierten Kaninchenherzen. Naunyn-Schmiedeberg's Arch. Pharmak. 1969 a. 265 236—237

Löffelholz, K. and E. Muscholl, A muscarinic inhibition of the noradrenaline release evoked by postganglionic sympathetic nerve stimulation. Naunyn-Schmiedeberg's Arch. Pharmak. 1969 b. 265 1—15

Löffelholz, K. and E. Muscholl, The effect of dexamphetamine on the output of noradrenaline from isolated rabbit hearts. Naunyn-Schmiedeberg Arch. Pharmak. 1970 a. 266 393—394

Löffelholz, K. and E. Muscholl, Inhibition by parasympathetic nerve stimulation of the release of the adrenergic transmitter. Naunyn-Schmiedeberg Arch. Pharmak. 1970 b 267 181—184

Malmfors, T. Studies on adrenergic nerves. The use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels. Acta physiol. scand. 1965. 64 Suppl. 248. 1—93

Miele E., Lack of effect of prostaglandins E and $F_{1\alpha}$ on adreno-medullary catecholamine secretion evoked by various agents. In "Prostaglandins, peptides and amines" Eds. P Mantegazza and E. W Horton, 85—93 Academic Press Lond. and N. Y. 1969

Montanari, R., E. Costa, M. A. Beaven and B. B Brodie. Turnover rates of norepinephrine in hearts of intact mice, rats and guinea pigs using tritiated norepinephrine. Life Sci. 1963 2 232—240

Oliverio A. and L. Stjärne. Acceleration of noradrenaline turnover in the mouse heart by cold exposure. Life Sci. 1965 4 2339—2343

Petersen P. V., N. Lassen, V. Hansen, T. Huld, J. Hjortkjaer, J. Holmblad, I. Møller Nielsen, M. Nymark, V. Pedersen, A. Jørgensen and W. Hougs, Pharmacological studies of a new series of bicyclic thymoleptics. Acta pharmacol. (Kbh.) 1966. 24 121—133

Potter L. T. and J. Axelrod, Studies on the storage of norepinephrine and the effect of drugs. J. Pharmacol. exp. Ther. 1963 140 199—206

Rosell, S., I. J. Kopin and J. Axelrod, Fate of H^3-noradrenaline in skeletal muscle before and following sympathetic stimulation. Amer. J. Physiol. 1963 205 317—321

Roth, R. H., L. Stjärne and U. S. v. Euler. Factors influencing the rate of norepinephrine biosynthesis in nerve tissue. J. Pharmacol. exp. Ther. 1967 158 373—377

Schümann, H. J., K. Starke and U. Werner. Interactions of inhibitors of noradrenaline uptake and angiotensin on the sympathetic nerves of the isolated rabbit heart. Brit. J. Pharmacol. 1970. 39 390—397

Sedvall, G. and J. Thorson, Adrenergic transmission at vasoconstrictor nerve terminals partially depleted of noradrenaline. Acta physiol. scand. 1965 64 251—258.

Sedvall, G. C., V. K. Weise and I. J. Kopin, The rate of norepinephrine synthesis measured in vivo during short intervals; influence of adrenergic nerve impulse activity. J. Pharmacol. exp. Ther. 1968. 159 274—282.

Sigg, E. B., L. Soffer and L. Gyermek, Influence of imipramine and related psychoactive agents on the effect of 5-hydroxytryptamine and catecholamines on the cat nictitating membrane. J. Pharmacol. exp. Ther. 1963 142 13—20

Sjöstrand, N. O. A note on the dual effect of prostaglandin E on the motor response of the isolated vas deferens to nerve stimulation. Experientia (Basel) 1971 (In press)

Starke, K. and H. J Schümann, Zur peripheren sympathikushemmenden Wirkung des Clonidins *Experientia* (*Basel*) 1971 *27* 70—71
Starke, K., U Werner R. Hellerforth and H J Schümann, Influence of peptides on the output of noradrenaline from isolated rabbit hearts. *E op J Pharmacol.* 1970. *9* 136—140.
Stjärne L., Studies of catecholamine uptake storage and release mechanisms. *Acta physiol. sc d* 1964 *62*. Suppl. 228. 1—97
Stjärne, L. and Å. Wennmalm, Preferential secretion of newly formed noradrenaline in the perfused rabbit heart. 1971 (T be publ.)
Thoenen, H., W Haefely and H. Staehelin, Potentiation by tetraethylammonium of the response of the cat spleen to postganglionic sympathetic nerve stimulation. *J Pharmacol. p Ther* 1967 *157* 532—540.
Trendelenburg, U Modification of the effect of tyramine by various agents and procedures. *J Pharmacol. xp Ther* 1961 *134* 8—17
Udenfriend, S and P Zaltzman-Nirenberg, Norepinephrine and 3 4-dihydroxyphenylethylamine turnover in guinea pig brain n vivo. *Science* 1963 *142* 394—396
Wennmalm, Å., P Hedqvist and L. Stjärne, Effects of hypothermia on norepinephrine release and effector response in isolated perfused cat spleen. *Circulat Re* 1970 *27* 39—44
Whittaker V P The isolation and characterization of acetylcholine containing particles from brain. *Biochem J* 1959 *72* 694—706
Vincenzi, F F and T C. West, Modification by calcium of the release of autonomic mediators in the isolated sinoatrial node *J Pharmacol. p Ther* 1965. *150* 349—360

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 366

From the Department of Physiology I Karolinska Institutet,
Stockholm Sweden

Isolation and Characterization of Sympathetic Nerve Trunk Vesicles

By
Hugo Lagercrantz

STOCKHOLM 1971

AB Egnellska Boktryckeriet, Stockholm 1971

Contents

The present survey is based on the following papers, except for some hitherto unpublished results:

1. Improvements in the isolation of noradrenaline storage vesicles from bovine splenic nerves. *Life Sci* 1970,9 639—650 (Together with R. L. Klein and L. Stjärne)
2. Highly purified noradrenaline storage vesicles from bovine splenic nerve trunk: Preliminary electron microscopy *Experientia.* 1970 26 994—995 (Together with A. Thureson-Klein, R. L. Klein and L. Stjärne)
3. Biochemistry of catecholamine storage: Some similarities between whole sympathetic nerve trunk vesicles and the membranes of adrenomedullary vesicles. *Acta physiol. scand* 1971 81 565—567 (Together with K. B Helle and L. Stjärne)
4. Lipids of the sympathetic nerve trunk vesicles. Comparison with adrenomedullary vesicles. *Acta physiol. scand 1971* In press.
5. ATP hydrolysis and noradrenaline transport in purified vesicles from splenic nerve trunk. *Acta physiol scand* 1971 In press (Together with R. L. Klein)
6. Unidirectional fluxes in isolated splenic nerve vesicles measured by a Millipore filter technique Effects of noradrenaline and competetive reversal of reserpine inhibition. *Acta physiol scand* 1971 In press (Together with R. L. Klein)

The papers will be referred to by their arabic numerals.

I Introduction

The present study has been performed at Prof. U.S v Euler's department at Karolinska Institutet. Under his leadership several members of the Department have concentrated much of their interest to the study of sympathetic neurotransmission. A good deal of the work has been concerned with catecholamine storage particles prepared from bovine splenic nerve Starting from an homogenate of this tissue Euler and Hillarp (1956) prepared a sediment by differential centrifugation, which contained about 20 % of the noradrenaline originally present in the intact nerves. This was the first successful demonstration that the amines in sympathetic nerves are stored in sedimentable particles. After some modification of the methods a preparation was found suitable for biochemical, physiological and pharmacological studies *in vitro* (see Euler 1958 1967 1969 Euler and Lishajko 1961 a, b 1963 a b 1967 a, b 1969)

Studies of these nerve trunk amine particles have led to the demonstration of several qualitative similarities to the adrenomedullary catecholamine storage particles. In both particles the amines are stored in a molar ratio to ATP of about 4 1 (Blaschko *et al* 1956, Falck, Hillarp and Högberg 1956, Schümann 1958) An ATP Mg^{++} dependent mechanism for amine uptake inhibited by reserpine, was demonstrated in both types of storage particles (Kirshner 1962, Carlsson, Hillarp and Waldeck 1963 Euler and Lishajko 1961 b 1963 c) Dopamine-β-hydroxylase (Kirshner 1957 Stjärne 1966) and chromogranin A (Blaschko and Helle 1963 Banks, Helle and Mayor 1969) have been found in the adrenomedullary and nerve trunk particles.

There are thus marked qualitative similarities between adrenomedullary and sympathetic nerve trunk amine particles Only for medullary particles have techniques as yet been worked out for separation from contaminating material this has allowed quantitative analysis of their chemical composition (Hillarp 1960 a, see Smith 1968) In view of the lack of data concerning nerve particles much of the present thinking concerning the chemistry of amine particles in sympathetic nerve terminals has been based on extrapolation from *in vitro* studies of adrenomedullary particles.

Differences with regard to release rate amine uptake and resistance to hypoosmotic lysis between the two types of vesicles were soon observed (Euler

and Lishajko 1961 a, 1963 b) followed by a critical comparison between the two types of amine storage particles by Stjärne in his thesis (1964) in which he emphasized the hazards of extrapolation from studies of the adrenal medulla to problems concerning the properties of sympathetic nerves. He has also questioned if the amine particles from splenic nerves trunk are similar in properties to those in the nerve terminals in adrenergically innervated organs (1968 1970) The major aim in the further studies of these problems was to purify amine storage particles from sympathetic nerve trunk and terminals, in order to make a quantitative comparison with adrenomedullary particles This should be of particular interest with regard to the hypothesis that secretion of neurotransmitter in sympathetic neurons as well as of hormones in adrenal medulla occurs by exocytosis (Douglas 1968 Smith 1968 1969 Smith *et al* 1970 Stjärne 1970 Geffen and Livett 1971) Accurate knowledge of the chemical composition of nerve terminal particles seems to be a prerequisite for final solution of this problem.

The immediate aim of the present study has been to make a preparation of nerve trunk particles of a defined degree of purity on the way to the much more difficult task to isolate nerve terminal particles and to study the chemical composition and functional properties of nerve trunk amine storage particles.

The project was initiated by Dr L. Stjärne and to a large extent carried out together with him and Dr R. L. Klein, who visited the department for one year

Nomenclature

Electronmicroscopical studies of the splenic nerve preparations showed characteristic granulated vesicles which were assumed to be the catecholamine storage particles (2) (Geffen and Livett 1971) As mentioned above, amine particles in the nerve trunk have been assumed to differ from those in the nerve terminals. The particles are thus in the present study called nerve trunk vesicles abbreviated to N trunk vesicles while those in the adrenal medulla are called A vesicles, and those from the sympathetic nerve terminals — N terminal vesicles.

Other abbreviations:
NA = noradrenaline
CA = catecholamine
ATP = adenosine triphosphate
CyOx = cytochrome oxidase
DBH = dopamine β-hydroxylase
TPPase = thiamine pyrophosphatase
MAO = monoamine oxidase
G-6-Pase = glucose-6-phosphatase
β-GPase = β-glycerophosphatase
SN = supernatant
SA = specific activity

II Isolation of amine storage particles (1)

Amine storage particles from bovine adrenal medulla can be isolated relatively pure by differential and density gradient centrifugation (Blaschko Hagen and Hagen 1957 Hillarp 1958) On electron microscopy the particles are seen as granulated vesicles with a diameter of 500—3500 A (Coupland 1965) The purest preparations are almost free from contamination with other subcellular particles (see Smith 1968 Helle *et al* 1971)

Amine storage particles from sympathetic nerves have been much more difficult to separate from other particulate material. By density gradient centrifugation N terminal vesicles from the rat heart were found to migrate with the microsomes (Potter and Axelrod 1963 a) A 60-fold purification of N terminal vesicles from the rat heart was achieved by Michaelson *et al* (1964) and Snyder Michaelson and Musacchio (1964) By a modified centrifugation technique combined with gel filtration a 1500-fold purification of N terminal vesicles from the rat heart was reported (Potter 1966) However the method was not described in detail, this degree of purification has not even been approached by other workers.

When bovine splenic nerve trunk was used as starting material conditions were much more advantageous, since these nerves consist mainly of sympathetic C fibres. After squeezing nerve trunks between nylon rollers or homogenization with the Ultra-Turrax apparatus and subsequent differential centrifugation of the press juice diluted with isotonic phosphate buffer a vesicle suspension was obtained in which about 30 % of the original NA was found, and which contained 300—1500 A vesicles (Euler and Swanbeck 1964 *cf* Euler and Lishajko 1967 b) In the experiments on amine release, reuptake and net uptake in nerve trunk vesicles Euler and Lishajko (1965 1967 b 1969) used homogenization of bovine splenic nerves followed by removal of crude material and larger particles by centrifugation 9 000 x *g* for 10 min. The supernatant containing a high proportion of the amine vesicles was used for the above mentioned studies. By combination of differential and density gradient centrifugation a sediment was prepared which contained, 1.5 µg/mg protein (Schümann and Burger 1970) Separation and characterization of different types of subcellular fractions obtained from bovine splenic nerves has been made recently (Hörtnagl, Hörtnagl and Winkler 1969 De Potter Smith and De Schaepdryver 1970)

The aim of this part of the present study was to make a preparation of N trunk vesicles defined by morphological and biochemical criteria.

Methodology

Experimental procedure

Splenic nerves were obtained 20-30 min *post mortem* and immediately chilled with ice. Usually 10—15 g nerves were dissected, minced and homogenized in an Ultra Turrax apparatus at a low speed for 2.5 min, all in cold. The medium used was 0.25 M tris-buffered sucrose pH 7 4—7 6 in the proportion tissue/medium of 1 2.5 (w/v) Other homogenization methods were tried such as Ultra-turrax operated at a high speed for a short time a motor driven teflon pestler glass homogenizer and squeezing between teflon rollers,how ever the present one was found to give the best final yield of particle-bound NA, and the highest NA/protein ratio

Homogenates were centrifuged at 600 g_{max} for 10 min to remove unbroken cells and nuclei. This initial step was omitted in certain experiments (4 5 6) The filtered supernatant or the initial homogenates were centrifuged at 10 500 g_{max} for 10 min (see Fig. 1) This type of centrifugation was found to be the most effective in order to get rid of as much CyOx activity as possible but to retain most of the particle-bound NA.

Density gradients were made by mixing 1.5 M sucrose D_2O solution with 0.25 M sucrose in 1/2 D_2O/ 1/2 H_2O The light sucrose solution was pumped at a constant rate into the heavy solution and mixed by magnetic stirring. The sucrose solution in this vessel, which became progressively more diluted with light sucrose was pumped at twice this rate into the centrifugation tube, resulting in the formation of linear gradients. In some experiments sucrose H_2O gradients were used for comparison, which were made in about the same way Later the method was modified (4 5 6) A short linear gradient ranging from 0.25 to 0 6 M sucrose was layered on a step consisting of 1.5 ml of 1.3 M sucrose-D_2O in order to get a better yield of NA particles in a small volume

The gradients with 4—5 ml of 10 500 g SN on top were centrifuged at 40 000 RPM for 90 min in a Beckman Spinco Ultracentrifuge (L2 65 B) using a SW 40 swinging bucket rotor Gradient fractions were removed with hooked pasteur pipettes, diluted to isotonicity and then centrifugated in a Ti 50 rotor at 226 000 g_{max} for 30 min. The pellets obtained were resuspended in tris-buffered 0.25 M sucrose (pH 7.2—7 4) Aliquots from the 10 500 g supernatant and pellet were centrifugated and resuspended in the same way to determine the recoveries of the analysed enzymes in the gradients.

Densities of the gradient fractions were measured by using micro spheres (Beckman, Palo Alto), or according to Oster (1965) by comparison between

aliquots of the gradient fractions with reference sucrose solutions in linear density gradients made of pertroleum and carbon tetroxide.

Analytical methods

The following constituents of the resuspended pellets from the density gradients were analysed.

NA Samples for NA analysis were stored in 0 4 M perchloric acid The amines were isolated by adsorption on alumina columns at pH 8 4—8 6 They were eluted with 0.3 M acetic acid and fluorometrically determined according to Euler and Lishajko (1961 c) A known solution of NA (1 μg/ml) was mixed with an aliquot of one of the samples and treated in exactly the same way and after correction used as standard.

Proteins were determined by using the Folin-Ciocalteus reagens according to Lowry *et al.* (1951) As a standard, bovine serum albumine was used. In some experiments the proteins were first precipitated with 0 4 M perchloric acid and then solubilized in 0.2 M NaOH.

Cy Ox as an indicator of mitochondrial contamination was measured polarographically at 30 C using a Clark oxygen electrode. The reaction mixture contained 0 1 m M cytochrome c, 16 mM ascorbate and 75 mM phosphate buffer at pH 7.5 in a total of 3 0 ml aqueous solution.

MAO as an indicator of contamination by the mitochondrial outer membrane was measured radiometrically according to Wurtman and Axelrod (1963) C^{14}-tryptamine was used as substrate

The following *phosphate hydrolysing enzymes* were analysed

G-6-Pase as an indicator of microsomal contamination, *β-GPase* as lysosomal marker and *TPPase* as indicator of Golgi vesicles and microtubules. All reactions were carried out in 3 0 ml at 37 C for 30 or 60 min after an initial equilibration for 10 min. The released inorganic phosphate was measured according to Post and Sen (1967)

Electron microscopy

The gradient fraction with the highest NA/protein ratio (F III) was diluted to isotonicity in 0.25 M phosphate buffered sucrose (pH 7.2—7 4) Samples were incubated for 15 min at 37 C to deplete the amine particles other samples were incubated under the same conditions but with the addition of NA (0.5 μg/ml) $MgCl$ (3mM) and ATP (3mM) to refill the amine particles (Euler and Lishajko 1963 b) Some samples were also taken directly from the cold gradient. All of them were layered over equal volumes of 6 % glutar aldehyde, with 0 154 M phosphate buffer (pH 7 2—7 4) in tubes, which

were centrifugated at 226 000 g for 30 min in the ultracentrifuge. Thus the vesicles were fixed in suspensions while being sedimented into pellets. The pellets were postfixed in 2 % OsO_4 containing the same phosphate buffer Grey to silver grey sections were cut with a diamond knife on an LKB Ultratome. The sections were stained for 15 min in 4 % uranyl acetate and 5—10 sec in lead citrate. Micrographs were taken with a Zeiss EM 9A.

Results and Discussion

A. Subcellular fractionation

Subcellular fractionation of the bovine splenic nerve homogenate was done by differential and density gradient centrifugation to isolate NA containing particles from other cell organelles. To quantify contamination the distribution of the most common subcellular marker enzymes were analysed. glucose-6-phosphatase (G-6-Pase) as a microsomal marker enzyme (see Reid 1967) cytochrome oxidase (CyOx) as a mitochondrial inner membrane enzyme, monoamine oxidase (MAO) as a mitochondrial outer membrane enzyme (see Ernster and Kuylenstierna 1969) β-glycerophosphatase (β-GP-ase) as representative for lysosomes (de Duve 1965) and thiamine-pyrophosphatase (TPP-ase) as a Golgi vesicle and a microtubular marker (Pellegrino de Iraldi and de Robertis 1970)

It soon appeared during the preliminary experiments that mitochondria and microsomes were the major contaminants in the nerve homogenate after removal of coarse particles and nuclei. 50 % of the CyOx activity could be removed by centrifugation at 10 500 g for 10 min, which was found to be optimal to get rid of as much CyOx activity as possible but still retain most of the NA.

The NA containing particles were found to be effectively separated from the microsomes by density gradient centrifugation. However the separation was not found to be satisfactory in conventional linear sucrose gradients, thus D_2O in stead of H_2O as solvent medium was tried. A comparison between these two solvent media demonstrated that by using D_2O the desirable separation was improved, hence sucrose D_2O-gradients were preferred.

The appearance of the sucrose D_2O gradient obtained after ultracentrifugation of the 10 500 g SN is illustrated in Fig. 1

Three bands were seen and fractionated. They were called F I, F II and F III in the order of increasing densities. F I occurred in the interface between the supernatant and the gradient in a thin concentrated band with a light orange staining. This band was separated by a thin translucent zone

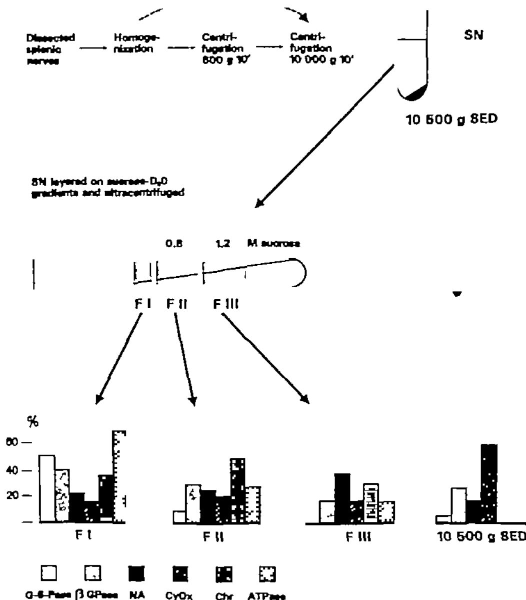


Fig. 1 *Differential and density gradient centrifugation of bovine splenic nerve homogenate.* NA amounts and enzyme activities are expressed in per centage recovered from the 600 g SN in the fractions; chromogranin A and ATPase per centage of the 10 500 g SN

from F II and F III which looked white and cloudy F II and F III were usually separated by a thin zone

F I was characterized by its high G-6-Pase activity and also by its high protein content. It was therefore regarded as a microsomal fraction. F II and F III contained high amounts of NA but less proteins. The relative specific activity of NA in relation to protein was found to increase about 2.3-fold in F II and 5 6-fold in F III, when compared with the original 10 500 g SN layered on to the gradients. F III is therefore called the N trunk vesicle pre paration (cf p 13) However also the lysosomal and mitochondrial marker enzymes were found to be enriched in F II and F III

B. The density of the N trunk vesicles

The density of F III was 1 16 g/cm^3 in the sucrose D_2O gradient, which was slightly higher than found in sucrose H_2O gradients (1 14 g/cm^3) The densities of F II and F III remained unchanged after ultracentrifugation for 5 hrs which indicates that density equilibrium was obtained.

Thus the density of N trunk vesicles increases in D_2O media compared to microsomes. In this respect N trunk vesicles resemble A vesicles, which become heavier in D_2O gradients (Poisner and Trifarò 1969 Laduron 1969)

In isotonic media, made of silica solutions N trunk vesicles seem to have about the same density as A vesicles (Lagercrantz, Pertoft and Stjärne 1970) Thus the differences in sedimentation properties between N trunk and A vesicles does not seem to be due to density Probably it is dependent on dif ferences in osmotic space, which is large in the A vesicles (Laduron 1969)

C. Distribution of NA

About 2/3 of the NA remaining in the original splenic nerve homogenate after removal of the 600 x g sediment could be recovered in particulate form. Of this about 1/5 occurred in F III, in sucrose D_2O gradients. The NA/protein ratio was 3 44 μg/mg (range 1 4—5 7) in the first series. On the basis of the finding that 67 % of the NA in whole nerves is sedimentable (i.e. about 0 07 μg NA/mg protein) the N trunk vesicle preparation represents a 50-fold (range 20—80) purification over the state in the isolated neuron.

By a slight modification of the gradients in order to get a higher N trunk vesicle yield a mixed fraction was obtained corresponding to F III and part of F II. The SA for NA in this fraction was somewhat lower 2.8 μg NA/mg protein (range 1 25—7 05 average of 52 determinations)

D Biochemical estimation of the degree of purity

With the exception of the study by Schümann and Burger (1970) earlier attempts to isolate N trunk vesicles by density gradient centrifugation have usually given only slight improvement with regard to NA/protein ratio (Burger Phillippu and Schümann 1969 Hörtnagl, Hörtnagl and Winkler 1969) compared to differential centrifugation only In these latter studies the particles contained in the high speed sediment were resuspended before layering on density gradients, this might cause some deletarious effects on the N trunk vesicles (cf Euler and Lishajko 1963 a)

By layering the 10 500 g SN directly on sucrose D_2O gradients some further improvement was achieved with regard to NA/protein ratio The use of D_2O instead of H_2O as a medium improved the separation between microsomes and N trunk vesicles, since the density of the N trunk vesicles was found to be slightly increased in D_2O

On the basis of calculation of the percentage distribution of NA, protein and the contaminating particles marked by the four enzymes studied (as representative of the total contaminants) in the various fractions, simultaneous equations were set up to be solved for that portion of F III which is NA vesicle protein The value was found to be approximately 18 % the remainder presumably representing contaminating material.

This can be regarded as a low estimate since it is based on the postulate by de Duve (1965) that each enzyme is entirely restricted to a single site within the cell. This might not be valid in this kind of tissue. The amine particles are assumed to originate from the Golgi membranes and it would not be surprising if they also retain some Golgi enzyme non-specific phosphatase activity such as β-GPase and TPPase (Pellegrino de Iraldi and de Robertis 1970) Furthermore it was later found that the method for CyOx determination to some extent yielded too high values in the N trunk vesicle fractions, presumably due to oxygen consumption mediated by DBH (see p 21)

E. Electron microscopy (2)

F III was further studied morphologically (Fig. 2) The N trunk vesicle preparation was fixed by layering F III diluted to isotonicity on glutaraldehyde in tubes which were centrifugated at a high speed (see p 9) The pellets obtained were found to consist of three major layers from the upper surface to about one quarter of the total pellet depth, essentially pure finely granular vesicles with a diameter of about 750 A were found below that layer vesicles became progressively less frequent and mixed with very dense osmiophilic granules and granular clusters in a thin zone in the bottom contaminants were found, mainly mitochondria. The granulated vesicles had the same ap-

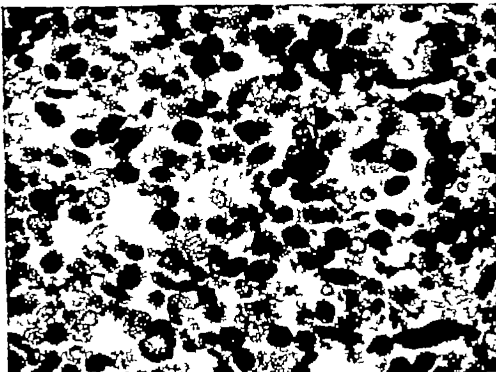


Fig. – *Isolated splenic erve trunk vesicles.*
F III was incubated with 0.5 µg NA, and Mg++ and ATP
This section is from the very pure upper layer of the pellet. Most of the vesicles seen are finely granular and have size of bout 750 Å. Fixation was carried out in 6 % buffered glutaraldehyde followed by postfixation in OsO_4. (This preparation was made together with Dr R. L. Klein and Dr Å. Thureson-Klein.)

pearance as those seen *in situ* with a diameter of about 750 Å they were surrounded by a 70 Å thick unit membrane

After incubation known to deplete the particulate NA these vesicles looked empty By incubation in ATP and Mg^{++} the matrix of the granulated vesicles became more prominent, and the free very dense osmiophilic clusters were less frequent.

The purity of the preparation was also judged by morphological criteria. The dominating finely granulated vesicles seen in the electron microscope were assumed to represent the NA storage particles, for the following reasons (*cf* Klein and Thureson-Klein 1971).

1 Large areas of vesicles similar in appearance could be found in the pellet of the purified N trunk vesicle fraction.

2 In size and morphology the vesicles were entirely similar to those seen in the axons of the starting material.

3 The density of the matrix material of the vesicles was altered by the same biochemical procedures which are known to fill them with, or to deplete them of NA.
4 The vesicles were strikingly similar in appearance to A vesicles although they were smaller in size.

Furthermore the vesicles observed in the present study are similar in size and in appearance to those which accumulate in the proximal stump of ligated axons to an extent which corresponds to the biochemically measured accumulation of NA (Kapeller and Mayor 1967 Geffen and Ostberg 1969)

The granular vesicles were found to represent between 40—60 % of all particles. On this criteria it appears that the estimation that the minimum purity was 18 % based on protein content in biochemical studies is too low: the figure should at least be doubled

This discrepancy between the biochemical and morphological estimation of purity can be rationally explained by the fact that in the first calculation it was assumed that all the enzymes measured were contaminants. As discussed above this is not certain. Furthermore the biochemical estimate gave only the lower limit for the relative proportion of NA storage particles.

It is a remote possibility that the granulated vesicles seen in F III are entirely unrelated to NA storage. However this does not seem likely since these particles would then have to represent some other cell organelle, not characterized by the marker enzymes analysed. The possibility of artifactual structures formed during the preparation and fixation procedure cannot be totally excluded but appears unlikely since similar results were obtained when the fixation procedure was varied (*cf* Klein and Thuresson-Klein 1971 unpublished observations)

F Comparison with N terminal vesicles

It cannot be assumed with certainty that the N trunk vesicles are the same as those which predominate in the varicosities. The N trunk vesicles prepared from bovine splenic nerve have a mean diameter of about 750 A (2, Klein and Thuresson-Klein 1971) while the NA particles seen in the N terminals have a diameter of about 450 A in different species (see Geffen and Livett 1971) Furthermore NA particles from innervated organs migrate to the microsomal or just below it in sucrose density gradients (Potter and Axelrod 1963 a, Potter 1966) while the N trunk vesicles have a higher density equilibrium (1) According to recent electron microscopial observations of homogenate of vas deferens analysed by sucrose density gradient centrifugation the "light" NA particles correspond to small granular vesicles, while the "heavy correspond to large granular vesicles (Bisby and Fillenz 1970) The denser

NA particles might at least in part originate from non-terminal axons in the organs. They may thus not be immediately concerned with transmitter secretion. This assumption is supported by the recent finding that prolonged nerve stimulation mainly depletes the small less dense vesicles (Kupferman, Gillis and Roth 1970 Bisby Cripps and Dearnaley 1971) However the granule properties and pattern in the vas deferens may differ from that in other adrenergic nerves (Euler and Lishajko 1966)

Summary

N trunk vesicles were isolated from bovine splenic nerve trunk by differential and density gradient centrifugation. According to biochemical criteria NA storage particles represent at least 18 % of the total particles in the gradient fraction having the highest NA/protein ratio. According to morphological evidence this estimation of the purity of the vesicle preparation is too low- it should probably be doubled.

The isolated N trunk vesicles have the diameter of about 750 A also seen in intact nerves. These vesicles are larger than the N terminal vesicles seen in the terminal varicosities. The N trunk vesicles were also found to have a higher apparent density equilibrium in sucrose gradients than that reported for N terminal vesicles. It can be concluded that N trunk vesicles with regard to morphology and sedimentation properties differ from N terminal vesicles.

III Chemical composition of nerve trunk vesicles

There are some indications that the structure of N trunk vesicles is basically similar to that of A vesicles since most of the characteristic constituents of A vesicles occur in amine vesicles from sympathetic nerves as well. CA and ATP in a molar ratio of about 4 (Schümann 1958 Euler Lishajko and Stjärne 1963 Potter and Axelrod 1963 a) chromogranin A (Hopwood 1968 Banks *et al* 1969) DBH (Potter and Axelrod 1963 a, Stjärne 1966, Geffen, Livett and Rush 1969 Hörtnagl *et al* 1969 de Potter *et al* 1970) Mg^{+} activated "ATPase" (Burger *et al* 1969)

While there are marked qualitative similarities between N and A vesicles, there are also marked quantitative differences. Thus to the crucial question of CA content calculation of the available data indicates that N terminal vesicles store CA at about the same concentration as the vesicles (see Folkow and Häggendal 1970) while the CA concentration of N trunk vesicles probably is much lower (Stjärne 1968) Similarly the CA.chromogranin ratio of A vesicles is 50 times higher than that in N trunk vesicles (Banks *et al.* 1970 Helle 1970) Furthermore N trunk vesicles have a considerably higher DBH activity in relation to CA content than the A vesicles (Hörtnagl *et al.* 1969) These facts, plus the striking differences in physical properties and in stability of CA storage in incubation in isotonic media at 20 —37° C and in sensitivity to osmotic changes and to drugs strongly suggests rather fundamental differences between N and A vesicles.

The marked differences in size sedimentation properties and in amine content between N trunk and terminal vesicles have been clearly recognized at this laboratory (Stjärne 1966, 1968 1970) Experiments have been carried out to solve the question how NA-poor "immature" N trunk vesicles are changed into NA-loaded "mature" terminal vesicles. The final solution apparently requires the successful isolation of N trunk and terminal vesicles for quantitative chemical analysis. As shown in the previous chapter a method has been worked out for routine isolation of N trunk vesicles of a defined degree of purity The present chapter deals with the analysis of the chemical composition of these N trunk vesicles.

NA particles might at least in part originate from non-terminal axons in the organs. They may thus not be immediately concerned with transmitter secretion. This assumption is supported by the recent finding that prolonged nerve stimulation mainly depletes the small less dense vesicles (Kupferman, Gillis and Roth 1970 Bisby Cripps and Dearnaley 1971) However the granule properties and pattern in the vas deferens may differ from that in other adrenergic nerves (Euler and Lishajko 1966)

Summary

N trunk vesicles were isolated from bovine splenic nerve trunk by differential and density gradient centrifugation. According to biochemical criteria NA storage particles represent at least 18 % of the total particles in the gradient fraction having the highest NA/protein ratio According to morphological evidence this estimation of the purity of the vesicle preparation is too low it should probably be doubled

The isolated N trunk vesicles have the diameter of about 750 Å also seen in intact nerves. These vesicles are larger than the N terminal vesicles seen in the terminal varicosities. The N trunk vesicles were also found to have a higher apparent density equilibrium in sucrose gradients than that reported for N terminal vesicles. It can be concluded that N trunk vesicles with regard to morphology and sedimentation properties differ from N terminal vesicles.

cal estimate of the degree of purity gave a lower figure 18 % As discussed in the previous chapter (p 13) this was a low estimate and based on the assumption that every marker enzyme belonged to the contaminants, which need not necessarily be true

Thus the true NA content in the N trunk vesicles from bovine splenic nerves could be estimated to about 10 μg/mg protein. This is strikingly low in comparison with the amine content of A vesicles where the amine/protein ratio is about 600 μg/mg (Hillarp 1959)

The present estimate of the NA content of N trunk vesicles is also much lower than the calculated amine content of N terminal vesicles (see Folkow and Häggendal 1970) which according to these authors should be about the same order as that of A vesicles.

In calculation of the life span of NA storage particles in sympathetic nerves Dahlström and Häggendal (1966) assumed that the NA content of N trunk vesicles is about the same as that of N terminal vesicles. It has been pointed out that such an assumption is not justified. Stjärne (1968) calculated on the basis of experimental data that the N trunk vesicles probably contain less than one per cent of the NA expected to be present in N terminal vesicles. This seems to be confirmed by the present findings.

It is not likely that the low figure for the NA content of N trunk vesicles obtained in the present study is due to NA loss during the experimental procedure. The studies were made in fresh material obtained within 30 min after the animals were killed. The time lag does not seem to be important, since comparison with nerves removed and chilled immediately after the animals were killed had only a slightly higher NA content (Lishajko, personal communication) Originally particle bound NA could also have been lost during the homogenization and centrifugations. However 2/3 of the total NA was recovered in the particulate fractions

Thus in spite of all these possible sources of error the NA concentration of N trunk vesicles in the intact nerve *in vivo* is probable less than 20 μg/mg protein this value is only 2—3 % of that found in A vesicles, and estimated in N terminal vesicles.

B Adenosine triphosphate (3)

The molar NA/ATP ratio was found to be about 4 in the N trunk vesicles, in accordance with earlier findings (see above) It follows that the ATP content was 9—18 μg/mg protein. This is considerably lower than the content of A vesicles. 430 μg/mg protein (Hillarp 1959) Recently it has been calculated that the NA/ATP molar ratio is in the range 7.5—12 in the N trunk vesicles with regard to mitochondrial contaminations (De Potter *et al.* 1970) It is

difficult to evaluate the significance of data obtained on the amine: ATP ratio in N terminal vesicle preparations since they are based on analysis of preparations probably heavily contaminated. However it is interesting that NA. ATP ratio of 2 9—4.2 and 1.3—3 7 have been found in rat heart particles (Potter and Axelrod 1963 a) and in rat vas deferens particles (Geffen and Livett 1971), respectively

C Chromogranin A (3)

Chromogranins were originally defined as the soluble proteins of A vesicles (Blaschko *et al.* 1967 a) Immunologically similar protein also occurs in N trunk vesicles (see above) However recent findings indicate that chromogranin-like material also occurs in a water insoluble form in A vesicles as in N trunk vesicles (Helle and Serck Hansen 1969 Banks *et al.* 1969 Helle 1970 1971 Mylroie and Koenig 1971) In this chapter only the best known quantitatively dominating chromogranin will be discussed, chromogranin A.

The protein of the N vesicle preparation (F III) investigated occured to only about 20 % in a water soluble form soluble chromogranin A was found only in trace amounts. This is distinctly different from the distribution found in A vesicles analysed by an identical technique soluble protein makes up about 80 % of the total proteins in these vesicles and chromogranin A represents 27 % of this fraction. Analysis of the waterinsoluble material of A and N trunk vesicles showed marked similarities with respect to chromogranin A content analysed by identical technique after osmotic lysis and extensive washing, they were solubilized in Triton-X 100 and assayed by the double diffusion technique. About 3 1 % of the N trunk vesicle protein was found to react with the chromogranin antiserum as compared to 21 % of the A vesicle "membrane" protein, in accordance with earlier findings obtained with similar technique (Banks *et al* 1969 Helle and Serck Hansen 1969 Helle 1971) This is in apparent contrast to the report of de Potter *et al* (1970) who found only 0 0026 mg chromogranin A/mg protein (0.26 %) in N trunk vesicles, and to that of Winkler *et al* (1970) who found only 0 04 mg chromogranin A/mg protein (4 %) in A vesicle membranes. However in these studies the membranes were exposed to repeated freezing and thawing, leading to considerable decline in protein/lipid ratio The chromogranin assay in these studies was carried out by micro-complement fixation technique this method is less suitable for determination of detergent solubilized proteins in view of the resulting unspecific hemolysis (Helle 1971)

The subcellular distribution of chromogranin A in the density gradient fractions of the N trunk vesicles preparation was about the same as that of NA. The NA/chromogranin ratio was found to be 620 nmoles/mg (310—

1150) in keeping with previous findings (Helle 1970 Banks and Helle 1971) which is considerably lower than that found in intact A vesicles (Schneider Smith and Winkler 1967) On the other hand it is strikingly close to the CA/ chromogranin A ratio in extensively washed membranes of A vesicles, which was found to be about 880 nmoles/mg (Helle, personal communication)

D Dopamine-β-hydroxylase (DBH) (3)

The DBH activity in the N trunk vesicles was found to be mainly membrane bound in accordance with previous reports (Hörtnagl *et al* 1969 De Potter *et al.* 1970) The specific activity was found to be very similar in the N trunk vesicle preparation (F III) to that found in the water-insoluble fraction of the A vesicles (see Table 1) In preliminary experiments an unexpected high DBH activity was found in the microsomal fraction (F I) when measured spectrophotometrically according to Gibb Spector and Udenfriend (1967) However when this was reinvestigated by the radiometric method using ^{3}H-tyramine as substrate (Friedman and Kaufman 1965 Viveros *et al* 1969) the DBH activity was found to have about the same distribution as the amines in the density gradients.

From the methodological point of view it is important to note that the observed DBH activities were altered by the presence of contaminating material, such as mitochondria (Flatmark, Helle, Lagercrantz and Stjärne to be published) A possible explanation is that cytochrome c might function as an electron donor to DBH (Flatmark, Terland and Helle 1971)

E Lipids (4)

Before the CA storage particles were detected, lipid binding of CA was investigated (Euler 1946, Norlander 1950) and discussed as a possible means for storage of the amines. After the discovery of the particles storing CA in stoichiometric relationship to ATP there was less obvious need for considering other mechanisms for amine storage. However Stjärne (1964) found evi dence that amines in the storage particles are stored in more than one form he pointed out that in addition to the storage of amines in a complex together with ATP there might exist a different form of amine storage, by binding to lipid or lipoprotein

Lipids may thus play a role in amine storage. In addition, the surprising finding of high amounts of lysolecithin in the A vesicles (Blaschko *et al* 1966), has drawn attention to the possibility that this lipid fraction may be involved in CA release at least in adrenal medulla.

The availability of a N trunk vesicle preparation of a high level of defined purity made it meaningful to carry out a lipid analysis of the purest fraction

the results were compared with these obtained in studies of A vesicles (Blaschko *et al* 1967 b) The N trunk vesicle preparation (F III) was found to contain somewhat more phospholipid (0 78 μmoles/mg protein) and cholesterol (0.35 μmoles/mg protein) than the A vesicles (see Table 1) The differences are not very marked and in fact they vanish if the values obtained are related to "membrane protein"[1]) The cholesterol/phospholipid ratio is of the same order 0 45 in the N trunk vesicle preparation and 0.58 in the A vesicle preparation (Blaschko *et al* 1967 b)

Lecithin and phosphatidylethanolamine were found to be the major phospholipids in N trunk vesicles as well as in the A vesicles. However only small amounts of lysolecithin were found in the N vesicles preparation. This phospholipid which occurs in a high concentration in A vesicles, making up about 15 % of the total lipids (Blaschko *et al* 1967 b) and has been ascribed a possible role for CA release from the adrenal medulla (see Smith 1968) The possibility remains that lysolecithin may be formed in the N vesicles on their way to the terminal varicosities, as a result of e.g. lysosomal phospholipase acting on the vesicle membranes, as suggested by Winkler and Smith (1968)

F ATP hydrolyzing enzyme (5)

Considerable interest has been devoted to the study of "ATPase" of the CA storage particles in chromaffin cells, since it was found that this activity is stimulated by Mg^{++} this is interesting in view of the fact that ATP Mg^{++} stimulate amine uptake in medullary particles (Hillarp 1958 Banks 1965 Kirshner 1966, Taugner and Hasselbach 1966 and Schümann and Burger 1969) and net uptake in N trunk vesicles (Euler and Lishajko 1963 b)

Measurement of ATP hydrolysis in the three gradient fractions (F I, F II and F III) in the present study showed that the SA of ATP hydrolyzing activity/mg protein was much lower in the N trunk vesicle fractions (F II and F III) than in the microsomal fraction (F I) in agreement with earlier findings (Hörtnagl *et al* 1969) In all fractions the ATP hydrolysis was found to be stimulated up to about 29—42 % by Mg^{++} in various combinations with NA^{+} and K^{+} The SA of Ca^{++} activated ATP hydrolyzing activity was also found to be about the same in three fractions however while K^{+} and Na^{+} tended to stimulate the activity in the microsomal frac

---

1) "Membrane protein of the N trunk vesicles — 45 % "Membrane protein of the A vesicles — 20 % assuming that the membrane is 70 Å and the mean diameter of the N trunk vesicle is 750 Å and that of the A vesicle 2000 Å.
The membrane protein was found to correspond to the water-insoluble protein in the A vesicles. However in the N trunk vesicles relatively more water-insoluble proteins (80 %) were found, possibly due to the preparation technique used.
Another explanation might be that the membrane of the N trunk vesicles is not organized as a unit membrane (cf Korn 1969).

Table 1
Biochemical constituents of the purified N trunk vesicle preparation The values are expressed per mg protein if nothing else is stated. The membrane-bound enzyme activities and chromogranin are expressed per mg water insoluble protein (wis). The individual phospholipids are expressed as per centage of total phospholipids. All the data concerning the A vesicles are from the review by Smith (1968), except the values for DBH and "ATPase" activities and the chromogranin amount (3 4).

	N trunk vesicles	*A vesicles*
CA	3 44 µg	600 µg
ATP	~85 µg	430 µg
Proteins	1 mg	1 mg
wis proteins	0.8 mg	0.2 mg
Chromogranin A /mg wis prot.	0.032 mg	0.25 mg
DBH/mg wis prot.	1 14 units	2.01 units
ATPase"/mg wis prot.	0.27 µM Pi	0.3 µM Pi
Lipids	1 mg	0.61 mg
Cholesterol	0.35 µmoles	0.25 µmoles
Phospholipids	0 78 µmoles	0.45 µmoles
Phosphatidyl-ethanolamine	31.8 %	36.1 %
Lecithin	36.6 %	26.0 %
Lysolecithin	3 1 %	16.8 %

tions, these monovalent ions produced significant inhibition in F II and F III

By further differential centrifugation of F III it was possible to remove most of the mitochondrial contamination. The basic Ca^{++}-activated ATP hydrolyzing activity was then found to be lower than that activated by Mg^{++} The former was no longer inhibited by K^{+} and Na^{+} and now the Mg^{++} activated hydrolysis was significantly stimulated by these ions. These findings agree to some extent with other studies of ATP hydrolysis in N trunk vesicles (Burger *et al.* 1969) and in isolated A vesicles (Banks 1965)

Since the amine uptake is known to be stimulated by ATP and Mg^{++} some attempts have been made to correlate "ATPase activity" with amine uptake. Reserpine has thus been reported to inhibit "ATPase" in amine vesicles and this has been regarded as a possible mechanism by which this drug blocks amine uptake. (Taugner and Hasselbach 1966, Burger *et al* 1969) However low concentrations of reserpine (10 —10^{-7} M) which effectively inhibited NA exchange did not affect the ATP hydrolysis The addition of NA or dopamine to the medium did not affect the ATP hydrolysis activity The data thus do not support the hypothesis of a link between ATP hydrolysis and NA transport in either direction across the membrane of isolated N trunk vesicles.

G. Electron transport and ATP synthesis in amine vesicles

The A vesicles contain a characteristic cytochrome, b-561 (Banks 1965 Ichikawa and Yamano 1965) This is of interest because it has been suggested that this cytochrome is a link in an electron transport chain to DBH which utilizes molecular oxygen (Flatmark, Terland and Helle 1971) This cytochrome has recently been found to occur also in the present N trunk vesicle preparation in two preliminary experiments (Flatmark, Helle, Lagercrantz and Stjärne, to be published) It was also noticed that the highly purified N trunk vesicle preparation (see 5) has a small oxygen consumption in the presence of dopamine (about 10 μg/ml) or ascorbate (*cf* Euler and Lishajko 1969 Giacobini unpublished observation) This oxygen consumption was about 10—20 % of that which was seen after the addition of cytochrome c. The findings may indicate the presence of an electron transport chain in N trunk vesicles, possibly related to cytochrome b-561 and DBH.

This electron transport chain demonstrated by Flatmark *et al.* (1971) may explain why metabolic inhibitors interfere NA uptake and release in isolated N trunk vesicles (Euler and Lishajko 1969) Whether this system can mediate an oxidative phosphorylation in the amine vesicles is not yet known. This seems to be an interesting possibility in view of the fact that A vesicles have the highest ATP concentration ever seen in subcellular structures (Blaschko *et al* 1956)

Another enzyme which is assumed to be able to provide the amine particles with ATP is adenylate kinase Such enzymatic activity has been detected in A vesicles (Hillarp 1958) However on reinvestigation of this question the low adenylate kinase activity in the A vesicle fraction appeared to represent mitochondrial contamination (Lagercrantz, Kuylenstierna and Stjärne 1970) The N trunk vesicles have not been studied in this respect, but there does not seem to be any reason to assume that this enzyme would exist in N vesicles but not in A vesicles. An additional possibility is that the amine vesicles may make their own ATP by a reversal "ATPase" reaction as in erythrocyte ghosts (Garahan and Glynn 1967)

Further biochemical studies of the amine particles are required to show how they are provided with ATP If they are not able to make their own ATP the most likely possibility is that the ATP originates from mitochondria. The problem is difficult to investigate in view of the low turn over of the intravesicular ATP in A vesicles. This is certainly the explanation why the ATP in the A vesicles was not labelled with administered P^{32} in two hours (Prusoff *et al.* 1961) However administration of P^{32} for one week effectively labelled the ATP of the A vesicles (Stjärne, Hedqvist and Lagercrantz 1970)

Summary

The present isolation of N trunk vesicles of a defined degree of purity has made it possible to compare N trunk vesicles with A vesicles with regard to chemical composition (see Table 1) The most striking difference is the low amine concentration in the N trunk vesicles compared with the A vesicles, 10—20 NA μg/mg protein *versus* 600 μg CA/mg protein. The A vesicles contain a large water soluble matrix, consisting of CA ATP and chromogranins. The corresponding soluble phase of the N trunk vesicles is much smaller

However the insoluble matrix of the two types of vesicles seems to be very similar (Fig. 4) They have about the same DBH and "ATPase" activities in relation to water insoluble protein. The cholesterol/phospholipid ratio was found to be about the same, and also the individual phospholipids, except that lysolecithin occurred only in trace amounts in the N trunk vesicles. These biochemical similarities between the insoluble phases also correspond to the amine and ATP binding capacities in isolated N trunk vesicles *versus* lysed A vesicles (Helle, Lagercrantz and Stjärne, to be published)

The present studies also indicate that N trunk vesicles differ from N terminal vesicles with regard to NA content. At present it is difficult to draw any other conclusions concerning the chemical composition of N terminal vesicles

However there does not seem to be any reason to assume that the N terminal vesicles are qualitatively different from the N trunk vesicles. It seems quite feasable that the N terminal vesicles may be developed from the N trunk vesicles by loss of some macromolecular material followed by subsequent loading with amines (Smith *et al* 1970)

IV Kinetics of amine uptake and release (6)

Isolated N trunk vesicles give off their NA at a high rate and are able to take up NA after the endogenous NA had been depleted by preincubation in a NA free solution and in that way become refilled. Two kinds of uptake processes were seen, one which is stereochemically fairly specific for 1-NA and is potentiated by ATP Mg^{++} (Euler and Lishajko 1961 a, 1963 b) and one which is not stereospecific and not ATP Mg^{++}-dependent. The latter kind of uptake can only be seen at high NA concentrations in the medium and may be a passive process.

The N trunk vesicles have many properties in common with the A vesicles such as an ATP Mg^{++} dependent CA uptake, which can be inhibited by reserpine (Euler and Lishajko 1961 b Kirshner 1962, Carlsson, Hillarp and Waldeck 1963) In addition to the difference in release rate the A vesicles cannot be refilled after partial depletion (Carlsson *et al* 1963 Lishajko 1969) as the N trunk vesicles. Furthermore the exchangeability of the CA in the A vesicles is only 20—30 % (Stjärne 1964 Taugner and Hasselbach 1966) while it is much higher in the N trunk vesicles (Stjärne 1964 Schümann and Burger 1970) Another important difference is that ATP is released along with the CA from the A vesicles, while in the N trunk vesicles the ATP loss is much smaller than the amine release (Euler Lishajko and Stjärne 1963 Stjärne 1964)

Since it has not been possible to isolate N terminal vesicles they have not been as extensively studied as those from the adrenal medulla and nerve trunk. However it was noted that the amine particles from heart were able to concentrate NA about 30-fold relative to the incubation medium. The uptake did not seem to be potentiated by ATP Mg^{++} or to be inhibited by reserpine (Potter and Axelrod 1963 b)

The words uptake and release are here used to describe the events seen irrespective of whether they are the results of exchange, active transport or a binding-unbinding process. Euler and Lishajko (1967 b) used the word "net uptake" indicating that uptake in excess of a concomitantly uptake and release occurs In order to study the "basic" release rate they excluded the effect of uptake by continuous removal of NA in the medium with ferri cyanide.

Another way to study "basic" NA uptake and release is to regard the *vesicles and the incubation medium as a closed two-compartment system* and analyse the data according to the general transport equation (Solomon 1953) This allows quantitative estimation of the unidirectional fluxes, and of the percentage of total vesicle bound NA which is readily exchangeable vesicle NA. A similar approach has been applied to the A vesicles (Taugner and Hasselbach 1966)

Methodology

To get a high yield of N trunk vesicles in a small volume the isolation procedure was modified as described earlier (p 8) the fractions F III and part of F II were condensed into a narrow band in the density gradients and then diluted to isotonicity K^+ and Na^+ ions were added usually as chlorides in order to approximate the K^+,Na^+ ratio naturally found in the axoplasm.

The basic vesicle suspension medium contained then. 20 mM tris-HCl buffer 50mM sucrose, 100 mM K^+ and 10 mM Na^+ and 10 μg/ml Pargyline, this drug was added to prevent metabolism of NA by MAO The suspension was incubated with tritium-labelled l NA (*NA) 1 ml aliquots of this suspension were removed at different time intervals (2, 5 15 30 45 and 75 min) and filtered through Millipore filters. The radioactivity in the filtrate, as well as that retained on the filters was determined in a Packard Tri-Carb liquid scintillation spectrometer Aliquots were also removed for determination of protein as well as for fluorimetric assay of NA.

The Millipore filters used (0 2 μ Celotate discs, Millipore Co Bedford) were found to retain 98.5 % of the N trunk vesicles. Dead space was measured in each experiment and the radioactivity trapped in this space was subtracted. Quenching was checked by internal standards.

Results and discussion

A. Analysis by a Closed Two Compartment System

Since no NA was lost during the incubation, the N trunk vesicles and their incubation medium represent a Closed Two Compartment System, allowing kinetic analysis according to Solomon (1953)

This analytical approach requires that the amines are distributed between two "compartments" with constant volumes, and that no loss of amines occurs. The incubation medium represents one compartment and the vesicles the other one.

The amount of labelled NA (represented by the radioisotope) in the vesicles at any time t is equal to the amount that enters (influx) minus the amount that leaves the vesicles (efflux) Influx is determined by the influx

IV Kinetics of amine uptake and release (6)

Isolated N trunk vesicles give off their NA at a high rate and are able to take up NA after the endogenous NA had been depleted by preincubation in a NA free solution and in that way become refilled. Two kinds of uptake processes were seen, one which is stereochemically fairly specific for l NA and is potentiated by ATP Mg^{++} (Euler and Lishajko 1961 a, 1963 b) and one which is not stereospecific and not ATP Mg^{++}-dependent. The latter kind of uptake can only be seen at high NA concentrations in the medium and may be a passive process.

The N trunk vesicles have many properties in common with the A vesicles such as an ATP Mg^{++} dependent CA uptake, which can be inhibited by reserpine (Euler and Lishajko 1961 b Kirshner 1962, Carlsson, Hillarp and Waldeck 1963) In addition to the difference in release rate the A vesicles cannot be refilled after partial depletion (Carlsson *et al* 1963 Lishajko 1969) as the N trunk vesicles. Furthermore the exchangeability of the CA in the A vesicles is only 20—30 % (Stjärne 1964 Taugner and Hasselbach 1966) while it is much higher in the N trunk vesicles (Stjärne 1964 Schümann and Burger 1970) Another important difference is that ATP is released along with the CA from the A vesicles, while in the N trunk vesicles the ATP loss is much smaller than the amine release (Euler Lishajko and Stjärne 1963 Stjärne 1964)

Since it has not been possible to isolate N terminal vesicles they have not been as extensively studied as those from the adrenal medulla and nerve trunk. However it was noted that the amine particles from heart were able to concentrate NA about 30-fold relative to the incubation medium The uptake did not seem to be potentiated by ATP Mg^{++} or to be inhibited by reserpine (Potter and Axelrod 1963 b)

The words uptake and release are here used to describe the events seen irrespective of whether they are the results of exchange, active transport or a binding-unbinding process. Euler and Lishajko (1967 b) used the word "net uptake" indicating that uptake in excess of a concomitantly uptake and release occurs. In order to study the "basic" release rate they excluded the effect of uptake by continuous removal of NA in the medium with ferricyanide.

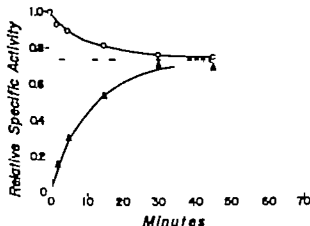


Fig. 3 *NA exchangeability of the N trunk vesicles*

Relative SA of N trunk vesicles and suspending medium *versus* time. Dashed line is the estimated steady state. Medium contained 0.5 µg 1-NA/ml, 3 or 5 mM ATP Mg^{++} 100 mM K^+ 10 mM Na^+ 50 mM sucrose, 20 mM tris-HCl buffer at pH 7.2—7.4 and 30 C. An average of 17 experiments.

according to equat. (4) The specific transfer coefficients can then be calculated by substitution into equations (6) and (7)

It was found that the influx coefficient is inversely related to the concentration of NA in the medium Conversely the efflux coefficient is directly related to NA concentration in the medium For example at a NA concentration of 0.5 µg/ml the NA influx coefficient is 650 times higher than the efflux coefficient. This ratio is reduced to 106 when NA in the medium is increased to 3.0 µg/ml. These changes are such that efflux balances the influx so the NA content of the vesicles is kept nearly constant.

Between 20 and 30° C Q_{10} for k_1 is about 2.8 and for k_2 about 2.4 at a NA concentration of 0.5 µg/ml, which indicates some sort of metabolically dependent process controlling both the influx and efflux of the transmitter This was previously assumed by Stjärne (1964)

It does not seem probable that both NA entry and exit in the vesicles can be governed by an active membrane transport. In addition, there is no evidence for a link between NA transport and ATP hydrolysis in these vesicles (5) The Q_{10} could be characteristic for the binding-unbinding process of NA from an intravesicular complex. This kind of a physico-chemical binding of some kind was suggested by Euler and Lishajko (1963 b)

D The action of reserpine

Not only the influx coefficient was diminished by low concentrations of reserpine but also the efflux coefficient was diminished. The most remarkable effect was seen on the exchangeability which was reduced to 5 % or less under these conditions. Even at a lower concentration of reserpine (2 × 10- M) the exchangeability was reduced 24 % while less effect was seen on the transfer coefficients.

It cannot be concluded, whether reserpine acts mainly on the membrane transport, on the binding of the amines in the vesicle matrix, or on both. The last-mentioned alternative is supported by earlier findings in this laboratory (Euler and Lishajko 1961 b Stjärne 1964) It was found that partially depleted N trunk vesicles were able to accumulate exogenous NA in the presence of reserpine, but not after preincubation of the vesicles with reserpine. Thus reserpine when acting over longer periods of time was assumed to "interfere with some factor necessary for the fixation of the CA to the granules" (Euler and Lishajko 1963 b) However these findings do not exclude that reserpine has its main action on the vesicle membrane.

The effect of reserpine could be reversed by raising the NA concentration. The inhibitory effects of 2×10^{-8} M drug were completely absent at a medium concentration of 3 0 μg NA/ml. The competitive nature of the inhibitory effects of reserpine on NA uptake has been reported earlier for A vesicles (Jonasson, Rosengren and Waldeck 1964) as well as for N trunk vesicles (Stjärne 1964) The latter author concluded that reserpine in order to competitively block uptake of amines occurring at 60—1000 times higher molar concentrations had to act "at some bottle-neck in the specific transport system of the granules" Since passage across the vesicle membrane did not seem to require specific transport he further assumed that this strategic site probably is located in the stroma of the interior of the vesicles This is partly supported by his finding that reserpine is inhibitory even at 0 C, while the present authors rather assume that these transport sites may be located in the vesicle membrane we agree that it is unlikely that reserpine acts on the intravesicular NA complex *per se* since reserpine in the 10^{-7} M range is able to completely and competetively inhibit exchange from an apparently homogenous vesicle pool of NA in the 5—10 mM range *i.e* at a molar ratio NA/reserpine of 50 000—100 000 (*cf* Taugner and Hasselbach 1966) Thus reserpine cannot block the amine sites stoichiometrically in the suggested binding-complex.

E. Comparison with the kinetics of the A vesicles

As mentioned above the N trunk vesicles are similar to the A vesicles in that they have an ATP Mg^{++} stimulated amine uptake which can be competitively inhibited by reserpine

However in several respects the two types of vesicles have different kinetic properties, which seem to be due mainly to differences in amine storage rather than in membrane properties (see Stjärne 1964 Taugner and Hasselbach 1966) The spontaneous amine release is much slower from the A vesicles and they cannot be refilled after partial depletion as the N trunk

vesicles. When the A vesicles are incubated they lose both CA and ATP at the same rate while the N vesicles lose mainly NA and not ATP In hypotonic media the A vesicles rapidly lose CA and ATP while the N trunk vesicles are much more resistant (Stjärne 1964) While some of these striking differences may be due to differences in surface/volume ratios, it seems that the main reason is that the amines in the A vesicles are predominantly stored in an unexchangeable, non-diffusible CA pool, which was not found in the N trunk vesicles. However the A vesicles have a smaller amine pool, which shows similarities with the amine pool in the N trunk vesicles. It is totally exchangeable and the amine "turn-over" appears to be within the same range as in the N trunk vesicles (Taugner and Hasselbach 1966)

It is possible that the amines in this small CA pool in the A vesicles are bound more loosely to the water-insoluble part of the A vesicles in an easily diffusible form while the large CA pool form an inner firmly bound complex with ATP and soluble proteins and earth metals (see Smith 1968) Since the N trunk vesicles consist to a larger extent of insoluble proteins and membrane it is likely that they do not contain this type of complex This was also suggested by Stjärne (1964) This might explain the differences in exchange ability sensitivity to osmotic shock and ATP release. It is further supported by preliminary kinetic studies of lysed A vesicles (Helle, Lagercrantz and Stjärne to be published) that these A vesicle "ghosts" behave in many ways as the N trunk vesicles. They have about the same amine binding capacity per mg protein, the CA are totally exchangeable, they have about the same CA. ATP ratio, and they have an ATP Mg^{++}dependent uptake, although there is *no net uptake.*

F Comparison with N terminal vesicles

The kinetic properties of N terminal vesicles have not been as extensively studied as those of other amine particles, mainly because they have not been isolated in reasonably pure preparations. According to data in the literature there seem to be some differences between N terminal and N trunk vesicles. The accumulation of exogenous NA in N terminal vesicles from rat heart was reported to be independent of ATP and Mg^{++} (Potter and Axelrod 1963 b) and not inhibited by low concentrations of reserpine. However it is possible that these apparent differences are mainly due to the impurity of the N terminal vesicle preparations used.

The rate of spontaneous release of NA from rabbit heart N terminal vesicles was about the same as that from bovine N trunk vesicles (Euler 1966) However on incubation under closely similar conditions N terminal vesicles from bovine spleen lose NA at a distinctly slower rate than N trunk

vesicles from bovine splenic nerves (Stjärne personal communication) It is further interesting that kinetic studies of one of the probably least contaminated N terminal vesicle preparation available, that from the short adrenergic neurons of the seminal vesicle of the bull, showed more similarities with the vesicles they had a slow spontaneous amine release and low amine uptake (Euler and Lishajko 1966)

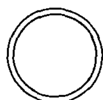
Summary

The present studies show that the amine pool in the N trunk vesicles is totally exchangeable at 20 and 30 C in a NA concentration range between 0.5 to 3 0 μg/ml The exchange seems to be due to a single component indicating the existence of one amine storage pool. The ratio between the influx and efflux coefficients is 650 at 30° C in a NA concentration of 0.5 μg/ml suggesting a strong tendency to take up NA. The Q_{10}s for influx and efflux transfer coefficients are in the 2.4—3 0 range, indicating that some kind of metabolically dependent process controlling both the influx and efflux of the transmitter It does not seem likely that both NA entry and exit are due to active transport, thus it is assumed that the phenomena seen could reflect a binding-unbinding process of NA from an intravesicular complex. This assumption is further supported by the finding that the influx coefficient was reduced while the efflux coefficient increased at higher NA concentrations in the medium.

However the possibility that the processes controlling influx and efflux involve active membrane transport cannot be excluded. The action of reserpine seems to be better explained by a membrane process than by an action on the storage complex. The main difference between A and N trunk vesicles seems to be that A vesicles have two main storage pools, one which is small and easily exchangeable, and one which is large and poorly exchangeable. According to the kinetic data in the present study all of the NA in the N trunk vesicles seems to be stored in one totally exchangeable pool.

A

N

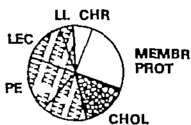


2000 Å

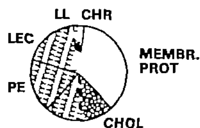
ⓐ

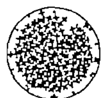


760 Å

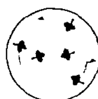


ⓑ





ⓒ

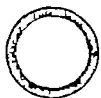


MEMBR PROT

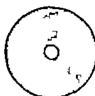
CA

ATP

CHR



ⓓ



EXCHANGEABLE CA POOL

c) While striking similarities were found between the water-insoluble constituentes of A and N trunk vesicles the latter store much less CA and ATP In the A vesicles the apparent CA concentration corresponds to about 0.55 M, compared with 0 005—0 010 M in the N trunk vesicles. As shown in the figure most of the CA are probably bound to ATP in the molar ratio of 4 or 3 An amine ATP complex at this ratio has recently been demonstrated to occur in the test tube (Berneis *et al* 1969) Amines may also be bound to an ATP-chromogranin A complex with a ratio CA.ATP of 3 1 (Helle 1971) Complexes between chromogranin A, ATP and CA may occur both in the soluble and in the insoluble phases (Banks and Helle 1971) Finally CA migth be stored independently of ATP possibly bound to phospholipids (Hillarp 1960 b Stjärne 1964 Euler and Lishajko 1967 b) In the figure all these possible types of amine binding are demonstrated without claims that the proportions are correct. However it can be assumed that a higher proportion of the amines in N trunk vesicles are bound in relation to the membrane phase than in the A vesicles.

d) Some indications about the storage of amines were given by the kinetic studies. In the A vesicles two amine storage pools have been detected. one large non-exchangeable and one small totally exchangeable. Preliminary studies indicate that there are similarities between the amine binding properties of lysed A vesicles and whole N trunk vesicles (Helle, Lagercrantz and Stjärne to be published) which suggests that most of the CA and ATP in the N trunk vesicles are probably stored in relation to the water insoluble protein, corresponding to the small CA pool in the A vesicles. The large amine pool in the A vesicles might be non-diffusible because of gel formation, with metal ions and chromogranins (Berneis *et al* 1969 see Smith 1968) This assumption is supported by the finding of concomitant loss of CA and ATP from A vesicles on incubation *in vitro* while ATP is largely retained in N trunk vesicles (see p 31) It can be noted that a less readily exchangeable amine pool was found in N trunk vesicles on incubation in a phosphate medium as indicated in the model by a small central core.

Fig. 4 CA storage in A and in N trunk vesicles.
) Relative sizes of an A vesicle (2 000 A) and N trunk vesicle (750 A). The relative membrane volumes are also demonstrated, assuming that the membrane is 70 A.
b) Chemical composition of the water-insoluble phases of the A and N trunk vesicles. The lipids are related to the membrane protein (see p. 22). LL = lysolecithin, LEC = lecithin, PE = phosphatidylethanolamine, CHOL = cholesterol, CHR = chromogranin A.
c) CA and ATP content in A and in N trunk vesicles.
d) Exchangeability of the CA in the A and in the N trunk vesicles. The white zones correspond t the nonexchangeable CA pools.

The role of N trunk vesicles in the secretion of NA

Valuable information on the mode of amine secretion from the adrenal medulla has been obtained by relating the nature of the material secreted from stimulated gland and the composition of the storage particles (see Douglas 1968 Smith 1969) How is this approach applicable in the case of neurotransmission secretion?

First of all it must be questioned if the investigated amine particles, the N trunk vesicles, are really involved in amine secretion from the nerve terminals. The present studies, in accordance with those of others (Stjärne 1968 1970 Geffen and Livett 1971) confirm the proposition that there are important differences between N trunk and N terminal vesicles. The former are larger and have a low amine concentration compared with the latter which are smaller and which according to calculation are assumed to have about the same high CA content as the A vesicles (see p 19)

Thus it must be assumed that the N trunk vesicles in some way are trans formed to N terminal vesicles. Earlier studies have shown that they have a high capacity to synthetize NA (Stjärne, Roth and Lishajko 1967) They may also be able to form ATP or to take up ATP from other sources in the terminal varicosities. It seems to be less probable that chromogranin is synthetized in the varicosities however it is possible that there may be a steady supply of soluble protein by solubilization of membrane protein under the influence of ATP as actually demonstrated by Helle (1971) This is further to some extent supported by the findings of morphological changes observed when N trunk vesicles are incubated with ATP and Mg^{++} (Klein and Thureson-Klein 1971)

Analysis of the perfusate from the stimulated spleen has demonstrated that the characteristic constituents of the N trunk vesicles chromogranin A and DBH are secreted along with NA (Smith *et al.* 1970) On the basis of these findings it was suggested that the amines are released by some kind of an exocytosis process. However the NA/chromogranin A and NA/DBH ratios were much higher than in the N trunk vesicles, and also in comparison with perfusate from stimulated adrenal medulla. Furthermore there is no indication of increased nucleotide release concomitant with the amine efflux (Stjärne, Hedqvist and Lagercrantz 1970) Therefore it has been assumed that neurotransmitter secretion occurs by some kind of "partial" exocytosis (see Smith *et al* 1970 Stjärne 1970 Geffen and Livett 1971)

The present results are compatible with this interpretation. N trunk vesicles are presumably precursors of N terminal vesicles and are apparently loaded with NA in the terminals. Since the membrane represents about 65 % of the total volume of N terminal vesicles it seems possible that a higher pro-

portion of the NA in these particles is bound in relation to the membrane phase, while a lesser proportion of the amines may be stored in a soluble non-diffusible amine complex, such as that in the A vesicles.

As proposed by Stjärne (1970) and Helle (1970) NA secretion may be initiated by exocytotic secretion of soluble amines, and macromolecules (ATP together with chromogranin A and DBH) from this small soluble amine pool Exposure of the membrane phase to immediate contact with the ions in the extracellular medium might lead to continued secretion of NA not accompanied by macromolecules. In spite of considerable reuptake of NA into the neuron, such a speculative secretion model may explain why the amine/chromogranin ratio in the effluent from the spleen is so much higher than in that from the adrenal medulla. After termination of secretion the vesicles might be functionally reconstituted by reuptake and *de novo* synthesis of NA and uptake of ATP (see p 24) and be reused a number of times (*cf* Smith *et al.* 1970). However there would be a progressive and irreversible loss of macromolecules. This might be the limiting factor determining the life span of the vesicles.

VI General summary

1 NA storage particles from bovine splenic nerves were separated from other particulate material by differential and density gradient centrifugation. According to electron microscopical evidence the preparation appeared to consist of NA storage vesicles to about 40—60 % Biochemical analysis of the purest fractions showed an about 50-fold increase in the NA/protein ratio over the original homogenate the preparation was free from microsomal contamination, but contained some lysosomes and mitochondria.

2 The NA/protein ratio of the purest fraction was found to be 3 44 μg per mg. Assuming that the preparation consisted of amine storage particles to about 40 % the NA/protein ratio in "pure" NA storage vesicles from sympathetic nerve trunk could be estimated to about 10 $\mu g/mg$, which is considerably lower than that found in isolated chromaffin amine storage vesicles, or that calculated to occur in nerve terminal vesicles. The NA/ATP ratio in the present preparation was found to be about 4

3 The present nerve trunk vesicle preparation showed about the same dopamine β-hydroxylase and Mg^{++}-stimulated ATP hydrolyzing activity as adrenomedullary vesicles. Chromogranin A was found mainly in the water insoluble phase and only in trace amounts in the water-soluble phase

4 The lipid composition of this preparation resembled that of adrenomedullary vesicles However only trace amounts of lysolecithin were detected in the preparation.

5 The kinetics of amine uptake and release were studied by a Millipore filter technique, which permitted analysis of unindirectional fluxes. In an ATP Mg^{++} supplemented medium at 30° and 20 C, the NA in the vesicles was found to be completely exchangeable with 0.5 to 3 μg 1 NA/ml in the medium. The kinetics are compatible with a single exponential component of exchange.

6 Reserpine (2×10^{-8} — 2×10^{-7} M) was found to "inactivate" the readily exchangeable NA pool, and to depress both the influx and efflux coefficients. The completeness of this effect was found to depend both on the concentration of the drug and on that of NA in the medium

7 The NA storage particles from nerve trunk show a number of similarities to the membrane phase of adrenomedullary vesicles. However they differ from intact adrenomedullary vesicles in content of amine, ATP and soluble chromogranin A. It is therefore assumed that they must develop into mature

terminal vesicles by accumulation of NA and ATP before they can function in transmitter secretion

Acknowledgements

I wish to express my deep gratitude to my tutor Dr Lennart Stjärne, who introduced me into the field of biogenic amines, and helped me with the experimental work and the writing of this thesis. Without his enthusiastic and encouraging support this study would not have been done.

I am likewise greatly indebted to Professor Richard L. Klein [illegible] Medical Centre U.S.A., with whom I had an extremely stimulating and [illegible] co-operation during the year (1969—1970) when he visited the [illegible]. Furthermore he was an excellent teacher for me in subcellular methods [illegible]

It has been an invaluble experience to work under Professor U. S. [illegible] direction. He has given stimulating advice and constructive criticism [illegible] the whole work.

I want especially to thank Dr Karen B. Helle, University of [illegible] for stimulating co-operation and discussions, and Dr Bo Kuylenstierna [illegible] Stockholm and Dr Håkan Pertoft, University of Uppsala [illegible] with biochemical problems.

The skilful technical assistance by Mrs. Ingrid Dahlin [illegible] son is gratefully acknowledged. My warm thanks are also [illegible] berg, who has typed the manuscript and to Mrs. Inga Norlander [illegible] the drawings.

The investigation was supported by Swedish Medical Research Council [illegible] 14X 2479-04B), Karolinska Institutet, Lars Hiertas [illegible] Sällskapet för Medicinsk Forskning

References

Banks, P The adenosine-triphosphatase activity of adrenal chromaffin granules. *Biochem. J* 1965 *95* 490—496.

Banks, P and K. B Helle, Chromogranins in sympathetic nerves. *Proc roy Soc* B. 1971 In press.

Banks, P K. B. Helle and D Mayor Evidence for the presence of a chromogranin-like protein in bovine splenic nerve granules. *Mol Pharmacol* 1969 *5* 210—212.

Bartlett, G R., Phosphorus assay in column chromatography *J biol Chem.* 1959 *234* 466—468

Berneis, K. H., A. Pletscher and M. Da Prada, Metal dependent aggregation of biogenic amines: A possible explanation for their storage and release. *Natur (Lond.)* 1969 *224* 281—283

Bisby M. A., H. Cripps and F Dearnaley Effects of nerve stimulation on the subcellular distribution of noradrenaline in the cat spleen. *J Physiol (Lond)* 1971 *212* 13—14 P

Bisby M A. and M. Fillenz, Isolation of two types of vesicles containing endogenous noradrenaline in sympathetic nerve terminals. *J Physiol (Lond.)* 1970. *210* 49—50.

Blaschko, H G V R. Born, A. D'Iorio and N R. Eade, Observations on the distribution of catecholamines and adenosine-triphosphate in the bovine adrenal medulla. *J Physiol (Lond)* 1956 *133* 548—557

Blaschko, H., R. S Comline, F H Schneider M Silver and A. D Smith, Secretion of a chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. *Natu (Lond)* 1967 a. *215* 58—59

Blaschko, H H. Firemark, A. D Smith and H. Winkler Lipids of the adrenal medulla. lysolecithin a characteristic constituent of chromaffin granules. *Biochem J* 1967 b. *104* 545—549

Blaschko, H., J M. Hagen and P Hagen, Mitochondrial enzymes and chromaffin granules. *J Physiol. (Lond)* 1957 *139* 316—322.

Blaschko, H. and K. B Helle, Interaction of soluble protein fractions from bovine adrenal medullary granules with adrenaline and adenosine-triphosphate. *J Physiol (Lond)* 1963 *169* 120—121 P

Burger A., A. Philippu and H. J Schümann, ATP-Spaltung und Aminaufnahme durch Milznervengranula. *Naunyn-Schmiedeberg's Arch. Pharmak exp Path* 1969 *262* 208—220

Carlsson, A., N A. Hillarp and B Waldeck, Analysis of the Mg++-ATP dependent storage mechanism in the amine granules of the adrenal medulla. *Acta physiol scand* 1963 *59* Suppl. 215 5—38.

Coupland, R. E., Electron microscopic observations on the structure of the rat adrenal medulla. I. The ultrastructure and organization of chromaffin cells in the normal adrenal medulla *J Anat (Lond)* 1965 *99* 231—254

Dahlström, A. and J Häggendal, Studies on the transport and life-span of amine storage granules in a peripheral adrenergic neuron system. *Acta physiol scand* 1966. *67* 278—288.

Douglas, W W Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Brit J Pharmacol* 1968 *34* 451—474

Duve, C. de, The separation and characterization of subcellular particles. *In* "The Harvey Lectures Series 59 Academic Press, New York and London. 1965 49—87

Ernster L. and B. Kuylenstierna, Structure, composition and function of mitochondrial membranes. *In* "FEBS symposium 1969 *17* 5—31

Euler U. S. v. A specific sympathomimetic ergone in adrenergic nerve fibres (sympathin) and its relations to adrenaline and noradrenaline. *Acta physiol. scand.* 1946, *12* 73—97

Euler U. S. v. The presence of the adrenergic neurotransmitters in intra-axonal structures. *Acta physiol. scand.* 1958, *43* 155—166.

Euler U. S. v. Release and uptake of noradrenaline in adrenergic nerve granules. *Acta physiol. scand.* 1966 *67* 430—440.

Euler U. S. v. Some factors affecting catecholamine uptake storage and release in adrenergic nerve granules. *Circulat. Res.* 1967 *20—21* Suppl 3 5—11

Euler U. S. v. Adrenergic neuroeffector transmission. *In* "The structure and function of nervous tissue Ed. E. H. Bourne. Vol. *2* 423—462. Academic Press, New York 1969

Euler U. S. v. and N. A. Hillarp, Evidence for the presence of noradrenaline in submicroscopic structure of adrenergic axons. *Nature (Lond.)* 1956, *177* 44—45

Euler U. S. v. and F. Lishajko, Noradrenaline release from isolated nerve granules. Acta physiol. scand. 1961 a. *51* 193—203

Euler U. S. v. and F. Lishajko, Effect of reserpine on the release of catecholamines from isolated nerve and chromaffin cell granules. *Acta physiol. scand.* 1961 b *52* 137—145

Euler U. S. v. and F. Lishajko, Improved technique for the fluorimetric estimation of catecholamines. *Acta physiol. scand.* 1961 c. *51* 348—355

Euler U. S. v. and F. Lishajko, Influence of pH on uptake and release of noradrenaline in adrenergic nerve granules. *J. Neurochem.* 1963 a. *10* 145—149

Euler U. S. v. and F. Lishajko, Catecholamine release and uptake in isolated adrenergic nerve granules. *Acta physiol. scand.* 1963 b. *57* 468—480.

Euler U. S. v. and F. Lishajko, Effect of adenine nucleotides on CA release and uptake in isolated adrenergic nerve granules. *Acta physiol. scand.* 1963 c. *59* 454—461

Euler U. S. v. and F. Lishajko, Effect of drugs on the storage granules of adrenergic nerves. *In* "Pharmacology of cholinergic and adrenergic transmission Proc. 2nd Int. Pharm. Meet. Prague 20—23 Aug. 1963 Czechoslovak Med. Press, Praha 1965 245—259

Euler U. S. v. and F. Lishajko, A specific kind of noradrenaline granules in the vesicular gland and the vas deferens of the bull. *Life Sci.* 1966, *5* 687—691

Euler U. S. v. and F. Lishajko, Mechanism of drug-induced catecholamine release from adrenergic nerve granules. *Circulat. Res.* 1967 a. *20—21* Suppl. 3

Euler U. S. and *F. Lishajko, Reuptake* and net uptake of noradrenaline in adrenergic nerve granules with a note on the affinity for l- and d-isomers. *Acta physiol. scand.* 1967 b. *71* 151—162.

Euler U. S. v. and F. Lishajko. Effects of some metabolic co-factors and inhibitors on transmitter release and uptak in isolated adrenergic nerve granules. *Acta physiol. scand.* 1969 *77* 298—307

Euler U. S. v. F. Lishajko and L. Stjärne, Catecholamines and adenosine triphosphate in isolated adrenergic nerve granules. *Acta physiol. scand.* 1963 *59* 495—496

Euler U. S. and G. Swanbeck, Some morphological features of catecholamine storing nerve vesicles. *Acta physiol. scand.* 1964 *62* 487—488.

Falck, B., N.-A. Hillarp and B. Högberg, Content and intracellular distribution of adenosine triphosphate in cow adrenal medulla. *Acta physiol. scand.* 1956, *36* 360—376.

Flatmark, T. O. Terland and K. B. Helle, Electron carriers of the bovine adrenal chromaffin granules. *Biochem. biophys. Acta (Amst.)* 1971 *226* 9—19

Folch, J. L. Ascoli, M. Lees, J. A. Meath and F. N. LeBaron, *Preparation of lipide extracts from brain tissue. J. biol. Chem.* 1951 *191* 833—841

Folkow B. and J. Häggendal, Some aspects of the quantal release of the adrenergic transmitter. *I.* "Bayer-Symposium II" Ed. H. J. Schümann and G. Kroneberg, Springer Verlag, Berlin-Heidelberg 1970. 91—97

Friedman, S. and S. Kaufman, 3 4-Dihydroxyphenylethylamine β-Hydroxylase. Physiol

Schümann, H J and A. Burger Calcium-Wirkungen auf die Noradrenalinspeicherung isolierter Milznervengranula. *Naunyn-Schmiedeberg s Arch. Pharmak.* 1970 *366* 185—194
Skipski, V P R. F Peterson and M. Barclay Quantitative analysis of phospholipids by thin-layer chromatography *Biochem. J* 1964 *90* 374—378
Smith, A. D Biochemistry of adrenal chromaffin granules. *In* A Symposium on "Interaction of Drugs and Subcellular Components in Animal Cells Ed. P N Campbell, J & A. Churchill Ltd. 1968 239—292.
Smith, A. D Macromolecules in synaptic function. *Neurosciences Res Prog Bull* 1969 *8* 377—382.
Smith, A. D., W P De Potter E. J Moerman and A. F De Schaepdryver Release of dopamine β-hydroxylase and chromagranin A upon stimulation of the splenic nerve. *Tissue & Cell* 1970 *2* 547—568
Snyder S. H., L A. Michaelson and J Musacchio, Purification of norepinephrine storage granules from rat heart. *Lif Sci* 1969 *3* 965—970.
Solomon, A. K., The kinetics of biological processes. Special problems connected with the use of tracers. *Advanc biol med. Phys.* 1963 *3* 65—97
Stjärne L., Studies of catecholamines uptake, storage and release mechanisms. *Acta physiol. scand* 1964 *62* Suppl. 228.
Stjärne, L., Studies of noradrenaline biosynthesis in nerve tissue *Acta physiol scand* 1966 *67* 441—454
Stjärne, L., *In* Ciba Foundation, Study Group no. 33 Adrenergic Neurotransmission Ed. G E. W Wolstenholme and M. O'Connor Churchill, London 1968. 113—115
Stjärne, L., Quantal or graded secretion of adrenal medullary hormone and sympathetic neurotransmitter *In* Bayer Symposium II, "New aspects of storage and release mechanisms of catecholamines Ed. H J Schümann and G Kroneberg, Springer Verlag, Berlin-Heidelberg 1970 112—127
Stjärne, L., P Hedqvist and H. Lagercrantz, Catecholamines and adenine nucleotide material in effluent from stimulated adrenal medulla and spleen. A study of the exocytosis hypothesis for hormone secretion and neurotransmitter release. *Biochem Pharmacol.* 1970 *19* 1147—1158.
Stjärne, L., R. H. Roth and F Lishajko, Noradrenaline formation from dopamine in isolated subcellular particles from bovine splenic nerve. *Biochem Pharmacol.* 1967 *16* 1729—1739
Strehler B. L. and J R. Totter, Determination of ATP and related compounds: Fire fly luminescence and other methods. *In* "Methods of Biochemical Analysis Ed. D Glick. 1954 *1* 341—356.
Taugner G and W Hasselbach, Über den Mechanismus der Catecholamin Speicherung in den "chromaffinen Granula des Nebennierenmarks. *Naunyn-Schmied berg s Arch Pharmak. exp Path.* 1966 *255* 266—286.
Viveros, O. H., L. Arqueros, R. J Connett and N Kirshner Mechanism of secretion from the adrenal medulla. III. Studies of dopamine β-hydroxylase as a marker for catecholamine storage vesicle membranes in rabbit adrenal glands. *Mol Pharmacol* 1969 *5* 60—68.
Winkler H., H. Hortnagl, H. Hörtnagl and A. D Smith, Membranes of the adrenal medulla. Behaviour of insoluble proteins of chromaffin granules on gel electrophoreses. *Biochem. J* 1970 *118* 303—310.
Winkler H. and A. D. Smith, Lipids of adrenal chromaffin granules: Fatty acid composition of phospholipids, in particular lysolecithin. *N aunyn-Schmiedeberg s Arch. exp Path. Pharmac.* 1968 *261* 379—388
Wurtman, R. J and J Axelrod, A sensitive and specific assay for the estimation of monoamine oxidase. *Biochem Pharmacol* 1963 *12* 1439—1441

SUPPLEMENTUM 367

From the Department of Histology Karolinska Institutet
Stockholm, Sweden

ACTA PHYSIOLOGICA SCANDINAVICA

Urban Ungerstedt

I. Stereotaxic mapping of the monoamine pathways in the rat brain

II. Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behaviour

III. Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system

IV Adipsia and aphagia after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system

STOCKHOLM 1971

Acta physiologica scandinavica
Supplementum 367

From the Department of Histology Karolinska Institutet, Stockholm, Sweden

Stereotaxic Mapping of the Monoamine Pathways in the Rat Brain*

By

Urban Ungerstedt

Abstract

The ascending monoamine pathways in the rat brain are demonstrated by the pile up of fluorescent material occurring in the axons after various types of lesions. The anatomy of the pathways is outlined in drawings of frontal sections of the brain and the origin and termination of several pathways is determined by studying the anterograde and retrograde degeneration occurring after well localized lesions. It is possible to separate the ascending NA pathways into a dorsal and a ventral bundle of axons. The dorsal bundle innervates the cortex and the hippocampus and the ventral bundle supplies NA nerve terminals to the medulla, the pons, the mesencephalon and the diencephalon. The dorsal bundle is found to originate in the locus coeruleus. Lesions of this nucleus abolish the nerve terminals in all cortical areas and in several other areas of the brain indicating a unique role for the locus coeruleus in influencing the activity of the entire brain The 5-HT pathways have a distribution similar to the ventral NA pathyway The course of the nigro-striatal and the meso-limbic DA pathways is presented in detail.

The following abbreviations are used Noradrenaline (NA) dopamine (DA) 5-hydroxytryptamine (5 HT) methyl-noradrenaline (α-m-NA) 6-hydroxydopamine (6-OH DA)

Introduction

In our studies on the central monoamine neurons we have experienced the need for detailed information on the course of the monoamine pathways in the rat brain. An atlas of the axon bundles would provide the necessary knowledge for making the monoamine neurons accessible with stereotaxic technique. We have therefore carried on experiments over a period of some years in order to arrive at a map of the monoamine pathways in the rat brain The results have been partly reported in some review articles during the course of the investigation (72 76—80) In the present article the results are presented in detail in the form of a series of frontal sections of the ascending monoamine pathways as well as an outline of the major individual monoamine systems.

Review of the literature

There is a vast literature related to the anatomy of the monoamine pathways in the brain. Today's knowledge derives from the results obtained by a number of different techniques such as biochemistry histochemistry electronmicroscopy and electrophysiology The following is a highly condensed review of the most important work in this field. Some of the results in the present investigation are confirming prior investigations with the same or different techniques and it is therefore regarded as important to present a reference list covering most of these studies. The character of the previous work has also set the aim of the present study an account of the localisation of the ascending monoamine axons and a knowledge of the principal organisation of the major monoamine systems.

The first evidence of possible monoamine neurons in the brain originate from the biochemical demonstration of NA (3 154) DA (26 40 94 122) and 5-HT (3 149) in various areas of the brain. The Falck and Hillarp histochemical technique provided the first proof of a cellular localisation of the monoamines (37 38 56) Subsequent work described the detailed localisation of the NA. DA and 5-HT cell bodies and nerve terminals (36 46 47 63 64 116) These studies have been extended to include different mammals (58 81 8? 106 114 115) and more careful studies of certain terminal areas like the cortex hippocampus and cerebellum (15 16 33 67 103) In order to determine the course of the different monoamine pathways lesion technique was used in connection with the fluorescence histochemical method and it was possible to describe the existence of descending NA and 5-HT pathways to the spinal cord (14 39 48) and ascending NA and 5-HT pathways from the brain stem entering the medial forebrain bundle (7 9 10 12, 13 50 51 118

119) The existence of a nigro-striatal DA system was proven in a number of studies combining biochemical and histochemical techniques (4 8 11 104 150) The tubero-infundibular DA system has also received much attention (18—22, 29 30 61 62, 68, 98 115 127 142) The description of a wide spread innervation originating in the locus coeruleus represented an important step in the more detailed analyses of the monoamine pathways (119) and the event of a new technique for demonstrating cortical NA nerve terminals (129) has made it possible to extend this investigation in this and previous papers to show that the locus coeruleus innervates all cortical areas of the brain (119 128, 152) While the 5-HT neurons are easy to separate from the catechol amine neurons by their yellow fluorescence it is impossible to separate the two catecholamine neurons from each other without special techniques such as spectrophoto-fluorometry (28 43) and pharmacology (35 47 63) When neurons are refered to as NA, DA or 5-HT neurons in the present study this refers back to the results from these investigations. It should be pointed out, however that recent evidence indicate that the neurons ordinarily referred to as 5-HT nurons might in fact be a heterogenous population containing true 5-HT neurons as well as neurons containing a not previously known monoamine (31 32)

Apart from the biochemical and histochemical identification of the central monoamine neurons it has recently been possible to detect them in the electron-microscope (34 52, 73 74 75 99—102) and with autoradiographical tech nique (105) The histochemical studies on the central monoamine neurons have been summarised in several review articles (65 66 69 70 71 72 76—80 92 93)

Apart from the histochemical studies related above there has been a number of important studies on the central monoamine neurons with biochemical technique, often in combination with experimental lesions or electrical stimulations. The existence of a nigro-striatal DA system has been inferred from measurement of DA levels in the human brain and from observations of the loss of striatal DA and nigral cell bodies in Parkinson s disease (54 87 88 94 95 96) as well as after experimental lesions in the ventro-medial tegmental area in animals (23 24 83—85 113 135 137—139 147 148) and in the globus pallidus (143) Similar lesions have also yielded histological (59 117) and electronmicroscopical (104) evidence for the existence of the DA path ways. Electrical stimulation in the region of the substantia nigra has yielded release of DA (121 140 141 155) as well as electrical responses in the caudate nucleus (41 42 60 97 156)

The ascending NA and 5-HT neuronal pathways have been extensively investigated by lesions and subsequent biochemical analysis. Lesions in various

areas of the mesencephalic tegmentum, the raphe area the medial forebrain bundle and the septum caused decreases in the levles of NA and 5-HT (25 27 53 86 89—91 106—110 120 123—126 131—134 136, 146) and also electronmicroscopical signs of degeneration within the 5-HT pathways (1) The most effective lesions damaged areas that corresponded closely to the areas where the ascending axons were found in the present study Electrical stimulation of the raphe region was shown to increase the turnover of 5-HT (2, 49 111 144 145) Stimulation of the spinal cord caused release of NA and 5-HT (5 6) and stimulation of various monoamine axon bundles caused an increased turnover of NA in the cortex and hypothalamus and DA in the striatum (17)

Material and Methods

There are a number of techniques to visualise the neuronal localisation of monoamines such as autoradiography enzyme histochemistry immunohisto-chemistry electronmicroscopy and histochemistry This investigation is limited to the Falck and Hillarp histochemical technique for monoamines (44 55 57 72) This method permits readily the visualization of NA, DA and 5-HT in cell bodies and nerve terminals while the axons contain too little monoamine to be detectable in the fluorescence microscope. Most of the techniques used in this investigation are therefore aimed at raising the content of NA DA or 5-HT in the axons. Besides this, anterograde or retrograde degeneration after lesions are used in order to find the origin and termination of a certain axon bundle

Histochemistry

The final analysis of the brains were made with the Falck and Hillarp histo-chemical fluorescence technique for monoamines (44 55 57 72) except in a few cases when the toluidin blue stain was used. The fluorescence method was carried out in principally the same way as described by Dahlström and Fuxe for the brain (47) However the careful tracing of monoamine pathways demanded certain changes in the procedure to minimise damage and facilitate anatomical orientation The animal was killed b decapitation during light chloroforme anaesthesia. The brain was quickly removed cut in four frontal sections which were placed on stiff pieces of paper A piece of nylon net was placed on the top of the tissue in order to prevent it from falling apart due to the cracks which develop during the freezing The brain pieces were frozen by dropping them into liquid propane cooled by liquid nitrogen and were then

transferred to an FT 1 freeze dryer (Bergman and Beving AB Stockholm) and processed for 2 1/2—3 1/2 days (130) After freeze-drying the brain pieces were removed by lifting the whole bottom plate of the freeze-dryer and transferred to a preheated desiccator A Petri dish with paraformaldehyde powder was placed in the bottom of the desiccator and formaldehyde gas was generated by placing the desiccator in an 80° C oven for 1 1/2 hour The bottom plate (with the brain pieces) was then transferred to a glass vacuum chamber and embedded for 10 min in liquid paraffine The pieces were sectioned in 10 μ sections, mounted and studied in a fluorescence microscope. The nylon nets and the easy transport of the brain pieces by the help of the bottom plate helped to preserve the anatomy of the brains.

From a number of brains, pieces of the cortex, the cerebellum or the hippocampus were removed bilaterally and smeared evenly on object glasses with the help of a cover glass. The brain smears were dried in the hot air stream from a hair drier placed in desiccators for 1 hour — 1 day and then reacted with formaldehyde gas as described above. This method is intended for the visualisation of monoamine nerve terminals in areas where they are usually difficult to detect, e.g in the cortex (129)

Stereotaxic technique

The animals were operated in a David Kopf stereotaxic instrument. Anaesthesia was induced by placing the animal in a glass jar and circulating a mixture of N_2O O_2 and halothane through the jar When asleep the animal was transferred to the head holder of the stereotaxic instrument where anaesthesia was maintained by circulating the same gaseous mixture through a mask fitted over the nose of the animal. A sagittal cut was made in the skin, the periosteum scraped off in order to disclose the anterior suture cross, i.e the bregma The tip of the coagulation electrode or the injection cannula was made to contact the bregma and its localisation was read from the instrument. The electrode or cannula was then moved a predetermined distance in the antero-posterior direction and in the lateral direction and a 2 mm wide hole was drilled with an electric trepan drill. Care was taken not to injure the surface of the brain. The tip of the electrode or cannula was brought in contact with the brain and the position was read from the instrument. The tip was then lowered to a predetermined depth. All movements of the instrument and all operative procedures on the scull were carried out using a stereoscopic operation microscope. An antibiotic powder was applied locally before suturing The animals usually woke up within 10 min after removing them from th instrument.

Hemisections of the brain were performed by lowering a blunt thin spatula, close to the midline until it reached the bottom of the brain. It was then gently moved in a lateral direction until the brain was completely sectioned on one side.

Intracerebral stereotaxic injections were made through a Hammilton cannula 0.2 mm in diameter. The cannula was connected to a motor driven "Agla micrometer all glass syringe" (Burroughs and Wellcome Co., England) by a PE 20 polythene tubing. The syringe was kept surrounded by ice to prevent the break down of the injected chemical. The fluid reached room temperature on its way through the tubing. Injection speed was 1 μl/min during intracerebral injections and 10 μl/min during intraventricular injections.

Electrocoagulations were made by radiofrequency current from a Siemens Radiotom. The electrodes consisted of pointed stainless steel needles 0.25 mm in diameter. The size of the coagulation was controlled by a number of variables. The size of the non insulated area at the tip of the electrode, the intensity of the current, the duration of the current and in the case of bipolar lesions the distance between the electrodes. Unipolar electrocoagulations were performed with the neutral electrode in the rectum, while bipolar electrocoagulations were performed after making the current symmetrical over an induction coil.

The size of the lesion was predetermined from the König and Klippel stereotaxic atlas (11?). The actual size was then tested by lowering the electrodes into a "model brain" consisting of egg white made solid by agar agar and measuring the size of the coagulated area through a measuring microscope. The solid egg white was kept in a cuvett which was brought to 37° C by keeping it in a thermostate controlled bath. The model brain is prepared as follows: 8 ml egg white is pipetted into a glass jar and placed in a 58° C water bath. ? ml boiling destilled water is pipetted into a glass jar containing 100 mg agar-agar. The agar-agar solution is heated until everything has dissolved. Care must be taken to minimize evaporation. The agar-agar solution is also placed in the 58° C water bath. When it has reached 58° C the egg white is poored into the agar-agar solution during careful stirring and allowed to stand in ?—4 minutes. The solution is then poured into cuvettes that are then sealed with Parafilm and turned upp-side down in order to force bubbles to the bottom. It solidifies in room temperature. When testing the size of a lesion the cuvett is placed in a 37° C water bath. When testing unipolar electrodes the neutral electrode is passed down the side of the cuvett. The size of the lesions are [illegible] measured through a dissection microscope. If the

TABLE I *Hemisections of the brain at different levels* First figures indicate total number of operated animals. Figures within brackets indicate the number of animals from which reliable drawings were made — animals killed after 1—2 days.

Level according to the König and Klippel atlas (mm)	Number of animals	Level according to the König and Klippel atlas (mm)	Number of animals
P 3.0	4 (2)	A 4 1	6 (3)
P 2.0	5 (2)	A 5 3	4 (4)
P 1 0	2 (1)	A 6.3	22 (4)
±0	3 (3)	A 7.3	16 (5)
A 1.0	3 (1)	A 8 4	9 (2)
A 2.4	2 (2)	A 9 4	18 (2)
A 3.2	3 (4)	A 10 0—11.0	20 (6)

"model brain" lesion is found to be smaller than the actual size in the brain, this can be corrected for by increasing the temperature of the bath to 38 C or 39 C. The opposite situation can be handled by lowering the temperature of the bath.

When the correct size of the lesion was arrived at in the model brain experiments by changing the parameters of the lesions, the size and location was tested in vivo by trypan blue infusion in the following way The coagulation was performed stereotaxically in the rat brain. While still under anaesthesia a 1 % solution of trypan blue was injected into the lingual vein in a volume of 10 ml/kg After 10 min the chest was opened and the heart cut open. The strongly coloured blood was then washed out by the infusion of saline through the lingual vein. The brain was removed, dissected and the localisation of the lesion was determined under an operation microscope The lesion was easily recognised by the trypan blue that had passed through the blood brain barrier at the site of the lesion.

Experiments intended to increase the fluorescence in the axons of the monoamine neurons

While the cell bodies and the nerve terminals of the monoamine neurons are easily visualized by the histochemical fluorescence technique the axons contain too low levels of monoamines to be detected and followed in serial sections. The most convenient way to increase the amount of monoamines in the axons is to lesion them and thus interrupt the axonal flow This results in a strong pile up of fluorescent material at the proximal side of the lesion (43)

TABLE II *Large electrocoagulations intended to produce pile up of fluorescent material in the monoamine axons — animals killed after 1—2 days*

Localisation (levels are indicated according to the König and Klippel atlas)	No. of animals
Reticular formation just medial to nucleus ambiguus	2
Reticular formation medial to the sensory trigeminal nucleus	2
Lateral to the fasciculus longitudinalis medialis just caudal to the inter peduncular nucleus	4
Medial forebrain bundle just rostral to the mamillary body (A 3 0)	6
Medial forebrain bundle at the level of mid hypothalamus (A 4.2)	6
Medial forbrain bundle at the rostral level of the nucleus paraventricularis (A 6 0)	4
Stria terminalis just caudal to the commissura fornicis (A 5.3)	3
Large bipolar lesions in the nucleus caudatus putamen destroying the whole caput and parts of the cauda	6
Nucleus accumbens (A 8.6)	6

The axons may then be tracted for several millimeters in serial sections. Three different ways of lesioning the axons were used in this study mechanical hemisections of the brain, electrocoagulations and localised injections of 6-OH DA.

Mechanical hemisections were performed at 14 different levels in different brains in order to visualise all ascending or descending pathways within the frontal planes. A total of 119 animals were operated. The number of animals are shown in table I

Electrocoagulations were made in the bundles revealed by the hemisection studies. Table II summarises the levels of the coagulations and the number of animals. The lesions were purposely made large in order to involve most axons.

6-OH DA injections were also performed into the bundles detected by the hemisection experiments as it is known that 6-OH DA lesions catecholamine neurons and produces extensive pile up of fluorescent material in the axons (150 151) Some injections were performed into terminal areas in order to visualise the afferent axons. Table III summarises the site of the injections and the number of animals. The doses were usually very high (20 μg/5 μl) in order to affect as many axons as possible. 6-OH DA was also injected intra

TABLE III *Injections of high concentration of 6-OH-DA intended to produce pile up of fluorescent material in the catecholamine axons — animals killed after 1—4 days.*

Localization (levels are indicated according to the König and Klippel atlas)	No. of animals
Lateral ventricle	10
Reticular formation, just medial to the nucleus ambiguus	3
Ventral to the locus coeruleus	4
Mesencephalic reticular formation, ventro-lateral to the aqueduct just caudal to the level of the interpeduncular nucleus (A 1.0)	8
Mesencephalic reticular formation, ventro-lateral to the aqueduct just rostral to the level of the interpeduncular nucleus (A 2.2)	6
Just rostral to the area ventralis tegmenti (A 2.4)	6
Medial forebrain bundle at the level of mid-hypothalamus (A 4.2)	
Amygdala (A 5.2)	5
Stria terminalis just caudal to the commissura fornicis (A 5.3)	2
Medial forebrain bundle at the level of the commissura anterior (A 7.2)	4
Nucleus caudatus putamen, caput (A 8.4)	6
Nucleus accumbens (A 8.6)	3
Septum (A 8.9)	3

ventricularly in a dose of 100 μg/20 μl. All the lesioned animals were killed 1 or 2 days after the lesion.

Another way to increase the fluorescence in the monoamine pathways is to inject α-m-NA into areas rich in monoamine nerve terminals and then follow the retrograde spread of the α-m-NA fluorescence in the axons (153). In this study α-m-NA (100 μg/10 μl) was injected into the corpus striatum of 8 animals that had received reserpine (10 mg/kg) about 24 hours before. They were killed 3—4 hours later and analysed with the histochemical fluorescence technique.

Degeneration studies

While it was possible to map the monoamine axonal pathways with the technique described in the preceding section other methods were needed to prove the origin and termination of the neuronal systems. Unilateral or bilateral electrocoagulations were performed in certain pathways or cell groups and after a period of at least 2 weeks the brains were analysed in order to

TABLE IV *Electrocoagulations intended to produce selective degenerations of certain monoamine pathways — animals killed after 10—14 days*

Localisation (levels according to the König and Klippel atlas)	No of animals
Locus coeruleus	10
A10 DA cell group just dorsal to the interpeduncular nucleus (A 1.8)	5
Caudo-lateral part of the A9 DA cell group in the substantia nigra (A 1.8)	4
Antero-ventral part of the A9 DA cell group in the substantia nigra (A 2.2)	4
Mesencephalic reticular formation, ventro-lateral to the aqueduct, just caudal to the level of the interpeduncular nucleus ("dorsal NA bundle") (A 1.0)	3
Mesencephalic reticular formation ventro-lateral to the above lesion ("ventral NA bundle") (A 1.0)	4
Both the above bundles (A 1.0)	10
Mesencephalic reticular formation, ventro-lateral to the aqueduct just rostral to the level of the interpeduncular nucleus ("dorsal NA bundle") (A 2.2)	21
Medial forebrain bundle at the level of the mamillary body (A 2.8)	6
Medial forebrain bundle at the level of mid-hypothalamus (A 4.2)	6
Medial forebrain bundle at the rostral level of the nucleus paraventricularis (A 6.0)	6
Stria terminalis just caudal to the commissura fornices (A 5.3)	4
Large bipolar lesions in the nucleus caudatus putamen destroying the whole caput and parts of the cauda, animals killed after 30—60 days	6

disclose the possible anterograde degeneration of certain nerve terminal systems or the retrograde degeneration of certain cell groups. The lesions were designed to involve specific pathways or cell groups and the size and localisation was carefully worked out by using the model brain and the trypan blue infusions (see above). The localisation of the lesions and the number of animals are shown in table IV.

Method of anatomical localisation of the monoamine neurons

The dark green background fluorescence in the microscopical sections revealed enough details to determine the exact relationship between certain brain

structures and the monoamine pathways. A special routin was adopted. The fluorescence microscope was equipped with a drawing attachement that permitted the observer to see the microscopical section together with his own hand, while making a drawing of the section. The drawing paper was illuminated from beneath and the intensity of the illumination was regulated by a thyristor arrangement. The illumination from beneath eliminated all disturbing shadows and the possibility to regulate the intensity of the illumination made it possible to mark the position of very weakly fluorescent structures. The drawings were then superimposed on the frontal planes of the König and Klippel atlas (112) and the exact localization of the pathways were determined.

Results

All h misections caused a pronounced pile up of fluorescent material that extended over 1—2 mm in the axons caudal to the lesions (fig. 3). A weak accumulation was also seen rostral to the lesions. However, the large necrosis made it sometimes difficult to obtain good microscopical sections that could be drawn with the help of the drawing attachment. Out of 119 brains 41 contributed sections that were easily drawn and photographed while the rest were seriously cracked or fragmented and could only be partly utilized.

The *electrocoagulations* produced the same kind of increase in the axonal fluorescence as the hemisection experiments but caused less bleeding and general damage (fig. 5—9).

The *intracerebral 6-OH DA injections* caused extensive pile up in the NA and DA axons while the 5-HT axons were unaffected (fig. 2). The axons were often followed for 3-4 mm in serial sections. The *intraventricular 6-OH DA injections* damaged most periventricular NA and DA nerve terminals (cf. 151) causing a strong pile up of fluorescent material in the axons entering the terminal areas.

The *a m. VA injections* produced an intense fluorescence in the head of the caudate nucleus and in the axons all the way back to the substantia nigra (fig. 2 and 4). Two populations of axons were distinguishable: strongly fluorescent axons that could be followed all the way from the substantia nigra to the corpus striatum and weakly fluorescent, more spread out axons, that did not enter the capsula interna.

The following sections will be devoted to a description of the anatomy of the monoamine pathways. The initial results were obtained from the hemisection experiments where all ascending pathways were interrupted. The pile up of fluorescent material occurring in the axons of these brains provided

detailed information of where the major axon bundles were located. The subsequent large electrocoagulations, 6-OH DA injections or α m-NA injections served to induce more extensive pile up in certain pathways. The final degeneration experiments intended to link certain cell groups or pathways to certain terminal areas and thus reveal the entire pathways.

The pathways will be described as they appear when all available information is brought together and the results obtained from a particular type of lesion will be presented only when it is necessary for evaluating the correctness of the conclusions.

Results obtained after increasing the axonal fluorescence by lesions or α m NA injections (letters within brackets refer to the drawings of the different frontal sections, the cell groups are labeled according to Dahlström and Fuxe (47))

At the level of the most caudal monoamine cell groups *A1 and A2* axons assembled in the mid-reticular formation to form an ascending bundle that received axons from the *A1* and in all probability from the *A2 cell group* (A) After intraventricular injections of 6-OH DA, the nerve terminals in the *nuc motorius n. vagi* and *tractus solitarius* degenerated and strongly fluorescent axons could be followed ventrolaterally towards the A1 cell group indicating that this nucleus provides axons to the terminals in these areas apart from sending descending (48) and ascending axons.

The ascending axons pass through the *radices n. facilis* receive fibres from the *A5 group* and occupy a position just medial to the *radices motoria n. facialis* (B) (cf fig 7)

The *A6 cell group (locus coeruleus)* sends axons laterally into the cerebellum (cf 128) caudally into the terminal areas in the medulla and medially where they cross the midline (C) The ascending axons from the locus coeruleus join the ventral ascending bundle and occupy the dorsal part of this bundle.

The *A4 cell group* (B) sends its axons towards the A6 group (C) The axons may join the rest of the ascending axons, enter the cerebellum or contribute terminals to the area medial to the *nuc tractus mesencephali n trigemini* which is densely innervated.

At the level of the *decussatio corporis trapezoidei* the ascending bundle receives axons from the *A7 cell group* (D) At the caudal level of the *nuc pontis* the axons in the dorsal part of the bundle bend dorso-medially (E) to form a distinct *dorsal bundle** (fig 1) and at the caudal level of the interpeduncular nucleus there is an obvious separation into a *ventral and a dorsal bundl* of ascending NA axons (F) The axons of the *dorsal bundle* assemble just latero-dorsal to the *fascic lus longitudinalis m dialis* to form

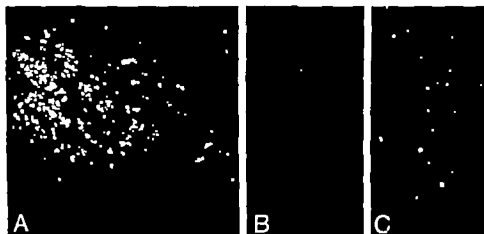


Fig 1 A. Pile up in the axons of the dorsal bundle after 6-OH DA injection f rther rostral. (× 100)

B. Smear from th cortex ipsilateral to dorsal bundle lesion. N terminal varicosities re seen. (× 300)

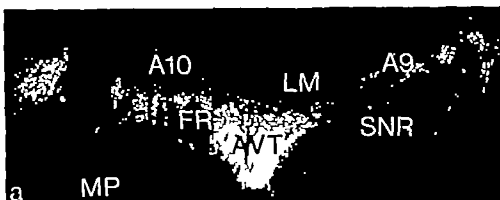
C. Smear from the contralateral cortex. The smear preparation is full of highly fluor escent ricosities. (× 300)

a tight bundle in which the axons are often assembled in small clusters (fig 1) The axons in the *ventral bundle* become spread out in an oblique band as they gradually turn ventro-medially along the *lemniscus medialis* (H) (fig 6)

Apart from the green fluorescent NA axons yellow fluorescent 5-HT axons emerge from the 5-HT cell bodies in the raphe region and form a distinct bundle of axons closely dorso-lateral to the *nuc interpeduncularis* (G H)

The DA cell bodies in the *zona compacta* of the *substantia nig a* (group A9) send their axons ventro-medially to join the axons from the DA cell bodies dorsal to the *nuc interpeduncularis* (group A10) whose axons leave the nucleus in an antero-lateral direction (H) (fig 2)

At th level of the caudal part of the *nuc mamillaris* there is a large number of ascending axons (I) The dorsal bundle of NA axons is running well separated while the rest of th axons are closely associated in the region of the *m dial fo ebrain bundle* Th ventro-medially and ventro-laterally located 5-HT axons (J N) are easily distinguished due to their yellow fluorescence while the NA and DA axons are difficult to separate. Their respective localization is deduced from the selective accumulation in the DA axons occurring after an electrocoagulation 6-OH DA injection or α-m-NA injec



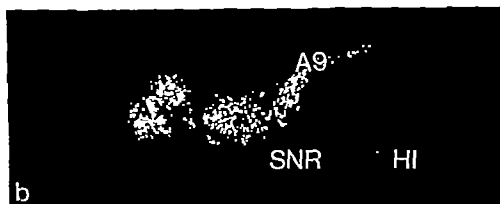




Fig 2. The ascending nigrostriatal DA neurons after injection f a-m-NA int th c *caudatus putam* The DA axons and th cell bodies show strong fluorescence due to retrograde transport of a-m-NA along the axons (× 30)

a. The A9 and the A10 cell groups send their axons towards the region of the *ar a vent alis t gment* (AVT) FR *fasciculus troflexus* LM *lemniscus m dialis* MP *mammillaris post* SNR *bstant gr con ticulat*

b. Rostral part of the right A9 cell group The DA axons (AX) hav formed distinct bundle. HI *h ppocampus* SNR *sub t tia gr eticulata.*



Fig 3. Unilateral hemisection of the brain. Mle p of fluorescence in the axons of the dorsal NA bundle and the ascending monoamine axons in the *medial forebrain bundle* (level A 2970, frontal section J) The dorsal bundle has turned entro-laterally to join the ascending monoamine axons (× 60)

tion into the *nuc caudatus putamen* and this is compared to the accumulation of NA and DA axons after hemisections or electrocoagulations of the *medial forebrain bundle* However being an indirect method the results are somewhat difficult to interpret and the bundles may occupy sligthly different positions compared to what is shown in the drawings.

The origin of the dorsal and the ventral NA bundle is partly revealed by the fluorescence accumulation occurring after lesions rostral or caudal to the *nuc interpeduncularis* Accumulations after lesions of the ventral bundle may be followed to a level caudal to the *locus coeruleus* while lesions of the

The meso-limbic DA axons (ML) are densely aggregated at the tip of the crus cerebri while the nigro-striatal axons have started to ascend in the capsula interna (CAI) towards the nuc. caudatus putamen (CP) (level A4620 frontal section M) TO *tractus opticus*.

ipsilateral cerebral and cerebellar cortex and the hippocampus (cf fig 1) Bilateral lesions caused a bilateral disappearance of the NA nerve terminals in these structures and also a decrease in the number of NA nerve terminals in most other areas of the brain especially in the brain stem nuclei. There was a minor decrease in the number of nerve terminals in the mesencephalon, the hypothalamus and the septal region. This is partly in contrast to the findings of Loizou (119) who found a more profound decrease in the hypothalamus. However Loizou might have been able to see more nerve terminals in the hypothalamus as he pretreated the animals with α-methyl DOPA to increase the terminal fluorescence. The nerve terminals originating in the locus coeruleus might be difficult to see in ordinary preparations from the hypothalamus in the same way as they are difficult to detect in the cortex.

A10 DA cell group dorsal to the nuc interpeduncularis

The lesions affected the nucleus bilaterally The rostrolateral part of the nucleus was probably not completely lesioned. However as the A10 and the A9 nucleus are continuous with each other this is difficult to ascertain (cf fig 2 A) The lesions caused a strong bilateral disappearance of DA nerve terminals from the nuc. accumbens and the tuberculum olfactorium

The lesions also severed the ascending 5-HT axons dorso-lateral to the nuc. interpeduncularis The disappearance of 5-HT nerve terminals were difficult to judge due to their weak fluorescence, however a considerable reduction was noted in the suprachiasmatic nucleus where the 5-HT terminals are easily seen.

Caudo-lateral and antero-ventral part of the A9 cell group in the substantia nigra

Lesions in the antero-ventral part including the area ventralis tegmenti caused a complete disappearance of the DA nerve terminals in the nuc. caudatus-putamen. Lesions of the caudo-lateral part denervated only the cauda and caudal part of the caput. This indicates that there is a corresponding topographical relationship between the cell bodies of the substantia nigra and their nerve terminals in the nuc. caudatus-putamen. The complete denervation of the nucleus after antero-ventral lesions was due to interruption of the axons from the more caudally located cell bodies.

Mesencephalic reticular formation

The hemisection experiments revealed that the ascending NA axons were separated in two distinct bundles at a level just caudal to the nuc. inter

peduncularis. Further rostral the ventral NA bundle mixed with the ascending DA axons while the dorsal NA bundle ran clearly separated from the other axons. Lesions of the dorsal bundle rostral or caudal to the nuc. interpeduncularis caused a disappearance of the NA nerve terminals in the ipsilateral cerebral cortex and hippocampus, while there was no clear reduction of the hypothalamic NA nerve terminals. There was also a strongly increased fluorescence in the ipsilateral cerebellar NA terminals indicating that the cerebellum is innervated by collaterals to the axons in the dorsal bundle (see Conclusion). Lesions of the ventral NA bundle caudal or rostral (fig. 5) to the nuc. interpeduncularis caused no decrease in the cortical or hippocampal nerve terminals but a strong decrease in the mesencephalic, the hypothalamic and the preoptic NA nerve terminals (fig. 8) and the NA nerve terminals in the nuc. interstitialis striae terminalis, ventral part (fig. 9). When both the dorsal and the ventral NA bundle were lesioned by one large lesion caudal to the nuc. interpeduncularis there was, as predicted from the results of the separate lesions, an almost complete disappearance of the ipsilateral NA nerve terminals from the forebrain. The cerebral cortex, the hippocampus and the nuc. interstitialis striae terminalis, ventral part were practically devoid of NA nerve terminals while there was a less complete disappearance in the ipsilateral hypothalamus indicating that a slight crossing might occur in the hypothalamus.

Medial forebrain bundle

Lesions of the medial forebrain bundle and the immediate surrounding brain parenchyma at the level of the mamillary body caused a disappearance of NA nerve terminals equivalent to what happens after a ventral bundle lesion (see above) and also a complete disappearance of the DA nerve terminals in the nuc. caudatus putamen, the nuc. accumbens and the tuberculum olfactorium.

Lesions of the medial forebrain bundle in the mid-hypothalamus caused the same degeneration of the DA nerve terminals provided that the lesion extended slightly into the crus cerebri. The nuc. dorsomedialis hypothalami and the surrounding areas showed normal innervation. The NA nerve terminals in the nuc. paraventricularis and the preoptic area were clearly diminished especially if the lesion extended medially. The nuc. interstitialis striae terminalis, ventral part, was denervated as well as the cortex and the hippocampus. The latter areas were involved because the dorsal bundle joined the ascending axons caudal to the lesion.

Large lesions in the rostral hypothalamus caused a slight decrease in the preoptic NA nerve terminals and a complete disappearance of the terminals

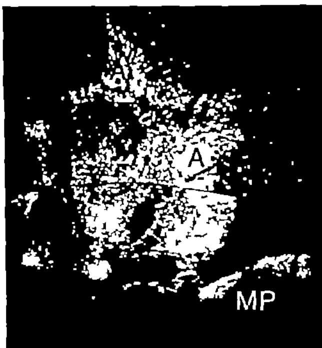


Fig 5. Small lectrocoagulation in the rostral *area vent lis t gmenti*. The ascending DA axons and the axons from the entral NA bundle are lesioned and show pile up (A) L lesion. MP: nuc. mamillaris posterior (× 100)

Fig 6 Pile p in the axons of the entral bundle just caudal to the A8 DA cell group (frontal section G after the lesion in fig 3 (× 160)

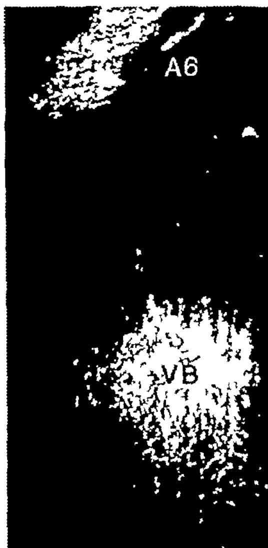
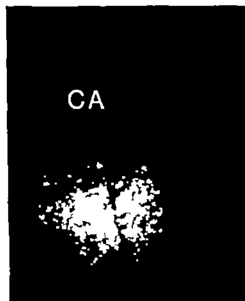


Fig 7 Pile up into the axons of the ventral bundle (*VB*) ventral to the *locus coeruleus* (A6) after the lesion in fig 5 The axons deriv from cell bodies caudal to the *locus coeruleus* (× 100)

in the nuc interstitialis striae terminalis, ventral part, as well as the DA nerve terminals in the nuc accumbens and the tuberculum olfactorium There was a strong reduction in the cortical nerve terminals but it seemed less pronounced as compared to the result after dorsal bundle lesions. However the axons are considerably spread out in the rostral hypothalamus, which was evident from



Fig. 8 A n paraventricularis after th lesion m fig 3 There is strong reduction in th NA nerv terminals ipsilateral to the lesion. (× 100)



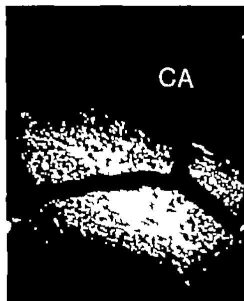


Fig 9 \ st is term alis tral pa t fter the lesion in fig 3 There is strong reduction in the number of nerve terminals on the lesioned side (left) while the control side show normal fluorescence (right) CA commissura anterior (× 100)

lesions restricted to the medial forebrain bundle which failed to denervate the nuc. accumbens and the tuberculum olfactorium and seemed to produce a less extensive degeneration in the cortex. The results may also indicate that axons leave the hypothalamus to enter the cortex caudal to the lesion. In one animal there was a retrograde pile up of fluorescence in axons that seemed to derive from the A13 group.

Striae terminals

A pile up of fluorescent material occurred in axons rostral to the lesion. There was no clear reduction of the DA nerve terminals in the nuc. amygdaloideus centralis, while there was a reduction in the density of NA nerve terminals in the amygdala area.

Large lesions in the nuc. caudatus putamen

The lesions destroyed the whole nucleus except the cauda. They extended slightly into the cortex, globus pallidus and lateral nuc. accumbens. There was considerable reduction in the number of DA cell bodies in the ipsilateral ventral mesencephalon. The DA cell bodies in the zona compacta of the substantia nigra (A9) the cell bodies surrounding the nuc. interpeduncularis (A10) and the cell bodies caudal to the substantia nigra dorsal to the lemniscus medialis (A8) were counted bilaterally in 12 sections from one animal covering all three nuclei. The number of cell bodies on the lesioned side were expressed as per cent of the number of cell bodies on the control side. In A9 there remained 25 % in A8 44 % and in A10 80 % of the DA cell bodies. The results indicate that the cell bodies in the zona compacta (A9) and probably the cells in the more caudally situated group (A8) innervate the nuc. caudatus-putamen while the cells surrounding the nuc. interpeduncularis (A10) innervate the nuc. accumbens and the tuberculum olfactorium as suggested by the lesions of the A10 group (see above) The 20 % decrease in the A10 might be due to the involvement of the lateral nuc. accumbens. The results from the other five animals corresponded to the above results, however no complete cell count were made in these animals.

In three animals there was a strong pile up of fluorescence in the nerve terminals in the hippocampus lateral to the substantia nigra. The lesion included the dorsal parts of the medial forebrain bundle in the preoptic region The increased fluorescence of the hippocampal nerve terminals were probably due to interruption of collateral to the axons innervating this part of the hippocampus (see Conclusions)

Conclusions

The monoamine neuron systems in the rat brain

On the basis of previous knowledge (see the review of the literature) and the experiments reported in the preceding sections of this paper it is possible to outline a number of separate monoamine neuron systems in the rat brain. In the following the origin and the termination of the systems are defined. The reader is also referred to the results of the hemisection experiments and the drawing of frontal sections (A—S) for their detailed anatomy. The cell groups are named according to the nomenclature of Dahlström and Fuxe (47)

Descending NA pathways (described in references no 48 included here for the sake of completeness)

The most caudal NA group A1 (and possibly A2) give rise to one system of fibres that descend in the anterior funiculus and the ventral part of the lateral funiculus and terminate in the ventral horn and another system that descend in the dorsal part of the lateral funiculus and terminate in the sympathetic lateral column and the dorsal horn. About half of the fibres to the sympathetic lateral column appear to cross the midline, while the fibres to the ventral and dorsal horn show much less crossing

Descending 5-HT pathways (described in references no 48 included here for the sake of completeness)

The most caudal 5-HT cell groups in the medulla oblongata (B1—B3) give rise to axons that descend in the medial part of the anterior funiculus and in the anterior part of the lateral funiculus to innervate the ventral horn and other axons that descend in the dorsal part of the lateral funiculus to innervate the dorsal horn and the sympathetic lateral column.

The ascending NA pathways from the lower brain stem—"the ventral NA pathway" (fig 10 12)

The A1 A2. A5 and A7 cell groups in the medulla oblongata and the pons give rise to one of the two major ascending NA systems. The axons ascend in the mid reticular formation (fig 7) turn ventro-medially along the lemniscus medialis (fig 6) and continue rostrally mainly within the medial forebrain bundle The system gives rise to NA nerve terminals in the lower brain stem, the mesencephalon and the diencephalon In the medulla oblongata and pons the ventral NA system and the other NA system originating in the locus coeruleus, overlap and contribute both to the terminal areas, how

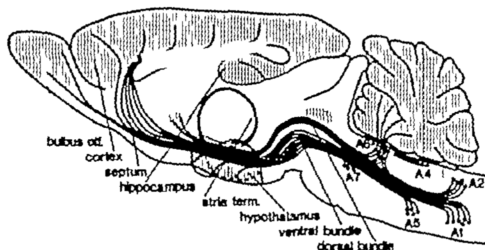


Fig 10. Sagittal projection of the ascending NA pathways. The descending pathways are not included. The stripes indicate the major nerve terminal areas.

ever. In the mesencephalon and the diencephalon the majority of NA nerve terminals seem to derive from the ventral system. A detailed information of the origin of the NA nerve terminals in the medulla oblongata and pons is still lacking due to the difficulties of making lesions in these areas and keep the animals alive for sufficiently long a time. However axons have been traced from the A1 cell group to the nuc. motorius dorsalis nervi vagi and the nuc. tractus solitarius. In the mesencephalon the ventral pathway has been traced to the major terminal areas i.e. the ventro-lateral part of the substantia grisea centralis and the part of the mesencephalic reticular formation dorsal and dorso-lateral to the lemniscus medialis at the caudal level of the interpeduncular nucleus. The ventral pathway innervates the whole hypothalamus most notably the nuc. dorso-medialis hypothalami, the nuc. periventricularis, the area ventral to the fornix, the nuc. arcuatus and the internal layer of the median eminence, the retrochiasmatic area, nuc. paraventricularis, nuc. supraopticus and the preoptic area. Further rostral the ventral pathway supplies the terminals in the densely innervated nuc. interstitialis striae terminalis, ventral part.

The ascending 5-HT system

The 5-HT cell bodies in the raphe nuclei especially the nuc. raphe dorsalis and the nuc. raphe medianus give rise to an ascending system of axons

that after running ventrally turn rostrally towards the interpeduncular nucleus The axons then occupy the most ventral part in the medial forebrain bundle and may be separated into a medial and a lateral component. The medial axons ascend in the septum and turn caudally in the cingulum. The lateral axons may leave the hypothalamus to enter the amygdala area. Due to weak fluorescence in the 5-HT nerve terminals it has been difficult to evaluate denervations after lesions and thus determine the areas innervated by the partways. However lesions of the 5-HT bundle dorsal to the inter peduncular nucleus has recently been shown to produce a strong decrease in the ability of cortical slices to take up ^{3}H 5-HT while the uptake of ^{3}H NA was unchanged (Farnebo Hamberger and Ungerstedt, unpublished data) This indicates that this bundle innervates the cortex.

Apart from sending ascending axons to the forebrain and descending axons to the spinal cord (see above) the raphe cells give rise to densely innervated areas in th lower brain stem. However details of the origin of this innervation are lacking.

Pathways originating in the locus coeruleus (fig. 10 12)

The NA cell bodies in the locus coeruleus give rise to a descending pathway that innervates the lower brain stem nuclei thus overlapping with the termination of the ventral NA pathway a lateral pathway that enters the cerebellum medial to the pedunculus cerebellaris medius (128) giving rise to the cerebellar NA terminals and finally an ascending pathway referred to in the following as the dorsal NA pathway Some axons from the locus coeruleus cross the midline medial to the nucleus These axons probably ascend, as bilateral lesions of the locus coeruleus are more effective in de nervating the hypothalamus (cf 118) The axons may also innervate parts of the lower brain stem. However the pathways to the cortices are uncrossed.

The dorsal NA pathway occupies the dorsal part of the ascending bundle of NA axons. At the caudal level of nuc pontis the axons turn dorso-medially to form a completel separated dorsal bundle of axons (fig 1) At the rostral level of the nuc mamillaris they turn ventro-laterally to join the ascending NA and DA axons fig 3) The axons then ascend in the medial forebrain bundle and in the septum and turn caudally in the cingulum. The pathway seems to gi e off branches to the corpora geniculata and the thalamic nuclei The lesions of th loci coeruleus and the dorsal bundle showed that this pathway innervates th cortex and the hippocampus (fig 1) However the way of entry into these areas is obscure Lesions in the rostral hypothalamus did not seem as effect in denervating the cortex as dorsal bundle lesions in the mesencephalon It seems probable that axons from the dorsal bundle

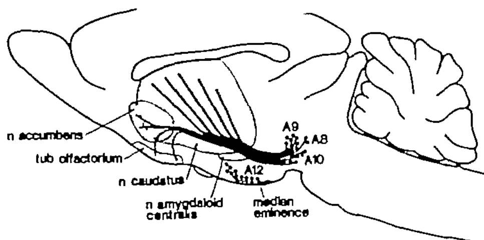


Fig 11 Sagittal projection of the DA pathways. The tripes indicate nerve terminal reas.

might leave the hypothalamus to enter the amygdala and the basal cortex in the commissura supraoptica dorsalis and in the region of the ansa lenticularis. This may constitute another entry into the cortex apart from the entry through the septal area. Parts of the hippocampal innervation probably also derives from direct hypothalamic connections while other axons may follow the cingulum caudally and then enter into the hippocampus.

The locus coeruleus gives rise to NA nerve terminals in practically all areas of the brain and especially in the cerebral and the cerebellar cortex and the hippocampus. Hence the nucleus is able to influence the whole brain in a unique way The fact that the cerebellar NA nerve terminals showed a strongly increased fluorescence ipsilateral to a lesion of the ascending axons from the locus coeruleus and that hippocampal NA nerve terminals showed increased fluorescence after lesions further rostral indicate that the same cell may innervate all three cortices. When one branch of the axon was lesioned the collaterals probably recei ed more storage granules due to the decreased total size of the axonal tree. This pattern of collateral innervation of all cortices has so far not been found in any other neuronal systems in the brain.

Th nigro-striatal DA system (fig 11)

The A9 DA cell group in the zona compacta and its rostro-medial extension in the area ventralis tegmenti gi e rise to a large pathway that ascend in the later

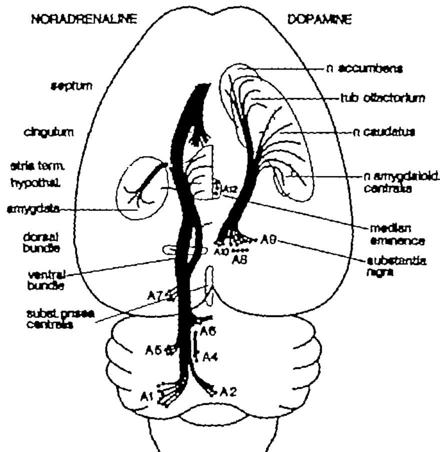


Fig 1.. Horizontal projection of the ascending NA and DA pathways.

al hypothalamus, enter the crus cerebri in the mid-hypothalamus, intermingle with the myelinated bundles in the capsula interna, fan out in the globus pallidus and enter the nuc caudatus putamen (fig 2, 4) The DA nerve terminals in the nuc amygdaloideus centralis seem to be an extension of the DA terminals in the nuc caudatus putamen and originate from axons running latero-ventrally from the capsula interna.

The axons from the A8 DA cell bodies caudal to the substantia nigra, dorsal to the lemniscus medialis, probably join the nigro-striatal DA system as they show retrograde degeneration after lesions of the corpus striatum.

The meso-limbic DA system (fig 11)

The axons from the DA cell bodies, dorsal to the nuc. interpeduncularis, ascend together with the axons of the nigro-striatal DA system. They follow

a more medial route and never enter the crus cerebri but ascend just dorsal to the medial forebrain bundle (fig 2 4) At the level of the commissura anterior one branch enter the nuc. accumbens and the nuc. interstitialis striae terminalis, dorsal part while another branch turn latero-ventrally to enter the tuberculum olfactorium from its more lateral aspect.

The tubero-infundibular DA system (fig 11)

The DA cell bodies in the hypothalamus are located within the nucleus arcuatus (A12) spread out along the lateral border of the periventricular nucleus and gathered in a dense group (A13) dorso-lateral to the nuc. dorsomedialis hypothalami pars dorsalis just ventral to the fasciculus mamillothalamicus. The A12 cells are known to innervate the external layer of the median eminence (37 61) The cells lateral to the nuc. periventricularis probably give rise to interhypothalamic DA nerve terminals while the A13 group might give rise to ascending axons as the group showed a retrograde pile up of fluorescent material after lesions of the medial forebrain bundle rostral to the cell group.

Acknowledgement

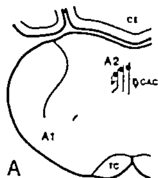
This work was supported by grant B71 14X 715-06C from the Swedish Medical Research Council and by grants from Svenska Livförsäkringsbolags Nämnd för Medicinsk Forskning and Ollie and Elof Ericssons Stiftelse I am greatly indebted to Miss Eva Henriksson and Miss Kerstin Sterner for their excellent technical assistance

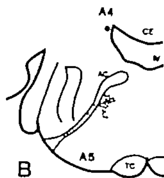
Frontal sections of the rat brain

The localisation of the monoamine axons are indicated in frontal sections of the rat brain. The drawings are based upon the König and Klippel stereotaxic atlas (112) and Craigie's Neuroanatomy of the Rat (157). The numbers refer to the distance in μ anterior (A) to the zero plane of the König and Klippel atlas. The localisation of the ascending NA, DA and 5-HT axons were determined by superimposing detailed microscopical drawings of the fluorescence microscopical sections on the König and Klippel atlas (see material and methods). The sections are marked by the letters A—S for easy identification. The cell groups are labeled according to Dahlström and Fuxe (47).

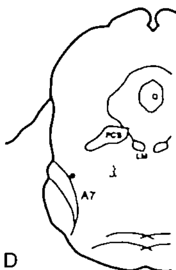
Index of abbreviations

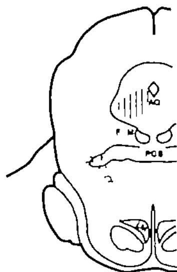
Abbreviation	Meaning
A	nuc. accumbens
AC	nuc. amygdaloideus centralis
AQ	aqueductus cerebri
C	cingulum
CA	commissura anterior
CAC	canalis centralis
CAI	capsula interna
CC	crus cerebri
CER	cerebellum
CO	chiasma opticum
CP	nuc. caudatus putamen
CPO	commissura posterior
F	columna fornicis
FL	fasciculus longitudinalis
FLM	fasciculus longitudinalis medialis
FMT	fasciculus mamillothalamicus
FR	fasciculus retroflexus
GP	globus pallidus
HA	nuc. anterior hypothalami
HD	nuc. dorsomedialis
HI	hippocampus
HV	nuc. ventromedialis hypothalami
IP	nuc. interpeduncularis
LM	lemniscus medialis
N FAC	nervus facialis (rad. motoria)
NM	nuc. mamillaris
PCS	pedunculus cerebellaris superior
PV	nuc. paraventricularis
SNR	substantia nigra, zona reticulata
ST	stria terminalis
SUT	nuc. subthalamicus
TC	tractus corticospinalis
TO	tractus opticus
TOL	tractus olfactorius lateralis
TUB	tuberculum olfactorium
VL	ventriculus lateralis
V IV	fourth ventricle

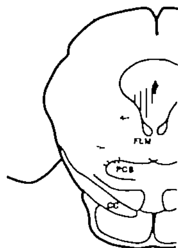

CE
A2
DCAC
A1
TC
A


A4
CE
AC
A5
TC
B


A6
C

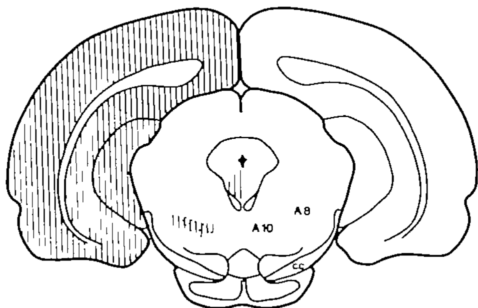

PCS
LM
A7
D



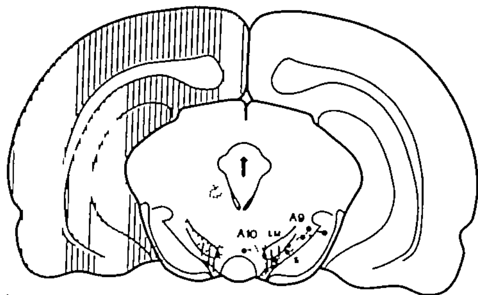


E A160

F A1020

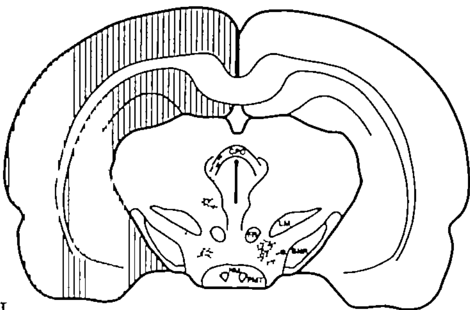


G A1270



H

A 1760

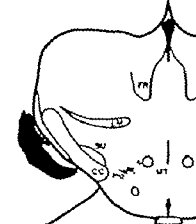


I

A 2580



J A2970

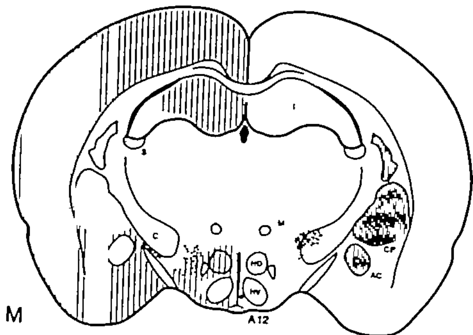


K A3430

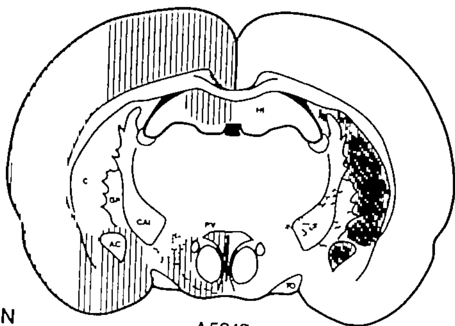
A13

A12

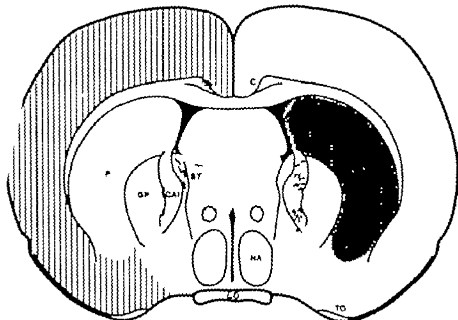
L A3990



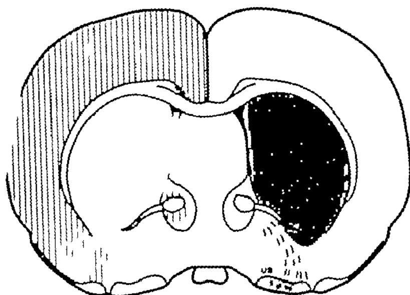
A4620



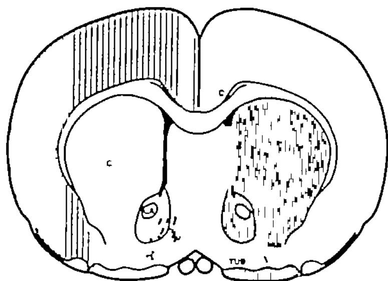
A5340

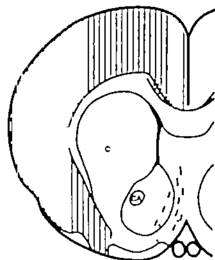


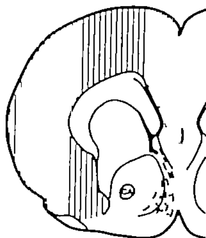
O A 6360



P A 7470


c
c
EA
TUB
Q
A8380


c
EA
R
A8920


EA
S
A9650

References

1 Aghajanian, G K., F E. Bloom and M. H. Sheard, Electron microscopy of degeneration within the serotonin pathway of rat brain. *Brain Res* 1969 *13* 266—273
2. Aghajanian, G K., J A Rosecrans and M. H. Sheard, Serotonin Release in the forebrain by stimulation of midbrain raphé. *Science* 1967 *156* 402—403
3 Amin, A. H., T B. B. Crawford and J H. Gaddum, Distribution of substance P and 5-hydroxytryptamine in central nervous system of dog. *J Physiol.* (Lond.) 1954 *126* 596—618
4 Andén, N E., A. Carlsson, A. Dahlström, K. Fuxe N A. Hillarp and K. Larsson, Demonstration and mapping out of nigro-neostriatal dopamine neurons. *Life Sci.* 1964 *3* 523—530
5 Andén N E., A. Carlsson, N A. Hillarp and T Magnusson, 5-Hydroxytryptamine release by nerve stimulation of the spinal cord. *Life Sci* 1964 *3* 473—478
6. Andén, N E., A. Carlsson, N A. Hillarp and T Magnusson, Noradrenaline release by nerve stimulation of the spinal cord. *Life Sci.* 1965 *4* 129—132
7 Andén, N E., A. Dahlström, K. Fuxe and K. Larsson, Mapping out of catecholamine and 5-hydroxytryptamine neurons innervating the telencephalon and diencephalon. *Lif Sci* 1965 *4* 1275—1279
8 Andén, N E., A. Dahlström, K Fuxe and K. Larsson, Further evidence for the presence of nigro-neostriatal dopamine neurons in the rat. *Amer J Anat.* 1965 *116* 329—334
9 Andén, N E., A. Dahlström, K. Fuxe, K. Larsson, L. Olson and U Ungerstedt, Ascending monoamine neurons to the telencephalon and diencephalon. *Acta physiol c d* 1966. *67* 313—326.
10. Andén, N E., A Dahlström, K. Fuxe, L. Olson and U Ungerstedt, Ascending noradrenaline neurons from the pons and the medulla oblongata. *Experientia.* 1966 22 44
11 Andén, N.-E., K. Fuxe B. Hamberger and T Hökfelt, A quantitative study of the nigro-neostriatal dopamine neurons system in the rat. *Act physiol and* 1966. *67* 306—312.
12. Andén, N E., K. Fuxe and K. Larsson, Effect f large mesencephalic-diencephalic lesions on the noradrenalin dopamine and 5-hydroxytryptamine neurons of th central nervous system. *Experientia.* 1966 22 842.
13 Andén N E., K. Fuxe and U Ungerstedt, Monoamine pathways to the cere bellum and cerebral cortex. *E perientia.* 1967 23 838.
14 Anderson E. G and L. O Holgerson, The distribution of 5-hydroxytryptamine and norepinephrine in cat spinal cord. *J Neurochem* 1966 *13* 479—485
15 Arbuthnott, G W A histochemical study of catecholamines in the somatosensory area of rat cerebral cortex. *J Physiol.* 1966 *186* 118—119
16. Arbuthnott, G W Noradrenaline uptake into cerebral cortex A histochemical study *J Neurochem* 1969 *16* 1599—1604
17 Arbuthnott, G W T J Crow K. Fuxe L. Olson and U Ungerstedt, Depletion of catecholamines *in vivo* induced by electrical stimulation of central monoamine pathways. *Brain R* 1970. *24* 471—483
18 Barry J Mise en évidence par stéréotaxie d transport hypothalamique t télencéphalique des monoamines élaborées a niveau d mésencéphale *C R Soc Biol*

1967 *161* 218?

19 Barry J Recherches sur l'origine et les sites de t rminaison des fibres monoaminergiques d tractus hypothalamo-hypophysaire *C R Soc Biol* 1968 *16* 1916.

20 Barry J Recherches morphologiques et expérimentales sur les cell les monoaminergiques d tractus hypothalamo-hypophysaire *An Endocr* (Paris) 1969 *30* 768—775

21 Barry J and J Leonardelli Étude de la topographie des neurones monoaminergiques au niveau de l'hypothalamus chez le Cobaye normal ou stéréotaxé *C R A ad Sci.* (Paris) 1967 *?65* 557—560.

22. Barry J and J Leonardelli Étude comparée des neurones et des fibres monoaminergiques de la région tubéro-infundibulaire chez le cobaye mâle normal ou castré *C R A ad Sci.* (*P ri*) 1968 *266* 15—17

23 Battista, A. F M Goldstein, N Nakatani and B. Anagnoste, The effects of ventrolat ral thalamic lesions on tremor and the biosynthesis of dopamine in monkey with lesions in the ventromedial tegmentum. *J Neu osu g* 1969 *31* *164—171*

24 Bédard P L. Larochelle A. Parent and L. J Poirier The nigrostriatal pathway A correlative study based on neuroanatomical and neurochemical criteria in the cat and the monkey *E p N ol.* 1969 *25* 365—377

25 Benetato, G R., M Uluity E. Bubuianu and C. Bonciocat, The effect of sectioning of the medial forebrain bundle and of sodium diethylbarbiturate on the content in catecholamines and serotonin of the hypothalamus and rhinencephalic structures (amygdala and hippocampus) *Rev. roum. Phy iol.* 1967 *4* 15—25

26. Bertler A. and E. Rosengren, Occurrence and distribution of catecholamines in the brain. *Act physiol. scand* 1959 *47* 350—361

27 Bertler A., B. Falck. C. O. Gottfries, L. Jungren and E. Rosengren, Some observations on adrenergic connections between mesencephalon and cerebral hemispheres. *A t pharmacol. et t xicol.* 1964 *21* 283—289

28. Björklund, A., B Ehinger and B F lck, A method for differentiating dopamine from noradrenaline in tissue sections by microspectrofluorometry *J Histochem. Cytochem* 1968. *16* 263—270

29 Björklund, A., A. Enemar and B. Falck, Monoamines in the hypothalamo-hypophyseal system of the mouse with special reference to the ontogenetic aspects. *Z Z llforsch.* 1968. *89* 590—607

30. Björklund, A., B. Falck, F Hromek, C. Owma and K. A. West, Identification and terminal distribution of the tubero-hypophyseal monoamine fibre systems in the rat by means of tereotaxic and microspectrofluorimetric techniques. *Brain R* 1970. *17* 1—23.

31 Björklund, A B. Falck and U Stenevi, Classification of monoamine neurons in the rat mesencephalon Distribution of new monoamine neuron system. *Brain Res* *1971* In press.

32. Björklund, A B. Falck and U Stenevi, On the possible existence of a new intraneuronal monoamine in the spinal cord of the rat. *J Pharmacol. p Ther* 1970 *175* 525—532.

33 Blackstad, T W K. Fuxe and T Hökfelt, Noradrenaline nerve terminals in the hippocampal region of the rat and the guinea pig *Z Zellforsch.* 1967 *78*, 463—473

34 Bloom, F E. and G K. Aghajanian, An electron microscopic analysis f large granular synaptic vesicles of th brain in relation to monoamine content. *J Pharmacol. p Ther* 1968. *159* 261—275

35 Butcher L., J Engel and K. Fuxe, L-Dopa induced changes in central monoamin neurons after peripheral decarboxylase inhibition. *J Pharm. Pharmac* 1970 22 313—316.

36. Carlsson, A., B Falck, K. Fuxe and N A. Hillarp Cellular localization f monoamines in the pinal cord. *Act physiol. scand* 1964 *60* 112—119

37 Carlsson, A. B. Falck and N A. Hillarp Cellular localization of brain monoamines. *Acta physiol. s and* 1962. *56* Suppl. 196 1—28

38 Carlsson A. B. Falck, N A. Hillarp and A. Torp Histochemical localization at the cellular level of hypothalamic noradrenaline. *Acta physiol. scand* 1962. *54* 385—386

39 Carlsson, A., T Magnusson and E. Rosengren, 5-hydroxytryptamine of th spinal cord normally and after transection. *Experientia*. Basel. 1963 *19* 359

40 Carlsson, A., M. Lindqvist, T Magnusson and B. Waldeck, On the presence of 3 hydroxytyramine in brain. *Scienc* 1958. *127* 471

41 Connor J D Caudate unit responses to nigral stimuli Evidence for a possible nigro-neostriatal pathway *Science* 1968 *160* 899—900.

4_ Connor J D Caudate nucleus neurones Correlation of th effects of substantia nigra stimulation with iontophoretic dopamine. *J Physiol.* 1970 *208* 691—703

43 Corrodi, H. and G. Jonsson, Fluorescence methods for the histochemical demonstration of monoamines. 4 Histochemical differentiation between dopamine and noradrenaline in models. *J Histochem. Cytochem.* 1965 *13* 484—487

44 Corrodi, H. and G Jonsson, The formaldehyde fl orescence method for the histochemical demonstration of biogenic monoamines. A review on the methodology *J Histochem. Cytochem.* 1967 *15* 65—78

45 Dahlström, A. and K. Fuxe, A method for the demonstration of monoamine containing neurons in the central nervous system. *Acta physiol. scand* 1964 *60* 293—294

46 Dahlström, A. and K. Fuxe, Localization of monoamines in the lower brain stem. *E perientia* 1964 *20* 398

47 Dahlström, A. and K. Fuxe, Evidence for the existence of monoamine-containing neurons in the central nervous system. I Demonstration of monoamines in the cell bodies of brain stem neurons. *Act physiol. scand.* 1965 *62* Suppl. 232 1—55

48 Dahlström, A. and K. Fuxe Evidence for the existence of monoamine neurons i the central nervous system. II Experimentally induced changes in the intra neuronal amine levels of bulbospinal neuron systems. *Act physiol. cand* 1965 *64* Suppl. 247 1—36.

49 Dahlström, A., K. Fuxe, D Kernell and G Sedvall, Reduction of th monoamine tores in the terminals of bulbospinal neurones following stimulation in th medulla oblongata *Lif Sci.* 1965 *4* 1207—121

50 Dahlström, A., K. Fuxe L. Olson and U Ungerstedt, Ascending systems of catecholamine neurons from the lower brain stem. *A ta physiol. d* 1964 *62* 485—486

51 Dahlström, A., K. Fuxe L. Olson and U Ungerstedt, On th distribution and possible function of monoamine nerv terminals in the lfactory bulb of the

rabbit. *Lif Sci* 1965 *4* 2071—20 4
5_ Descarries, L. and B. Droz, I traneu al distribution of exogenous norepinephrine in the central nervous sy tem of the rat. *J C ll Biol* 1970. *44* 385—399
53 Donoso, A. O Effect of brain lesions on hypothalamic noradrenalin m r ts. *Ex perentia*. 1966, *2* 191
54 Ehringer H. and O Hornykiewicz V rteil ng on Norad enab und Dopamin (3-Hydroxytyramin) im Gehirn des Menschen und ihr Verhalten bei Er krankungen des extrapyramidalen Sy tems. *Kl Il ch* 1960 *38* 1236—1238
55 F lck, B., Observations of the pos billties of the cellula localization of monoamines by a fluorescence method. *A ta physiol. scand* 196 *56* Suppl. 197 1—76.
56. Falck, B and N A. Hillarp, On the llular localization of techolamines in tl e brain. *Acta anat*. 1959 *38* 277—279
57 Falck, B N A. Hillarp G Thieme and A. Torp, Fluorescence of catecholamines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962. *10* 348—354
58. Falck, B L. Ljungrren and L. Nordgren, Diencephalic catecholamines i chick and pigeon. *Lif Sci*. 1969 *8* 889—893
59 Faull, R. L. M. and J B. Carman, Ascending projections of the substantia nigra in the rat. *J comp Neu ol*. 1968. *132* 73—92.
60. Frigyesi, T L. and D F Purpura, Electrophysiological analy n f reciprocal caud to-nigral relations. *B ai Res* 1967 *6* 440—456
61 Fuxe, K., Cellular localization of monoamines in the media eminence nd i in- fundibular stem of some mammals. *Acta phy iol sc d* 1965 *58* 383—381
62. Fuxe K., Cellular localization of monoamines in the median eminence and in- fundibular stem of some mammals. *Z Zellforsch* 1961 *61* 710—724
63 Fux K., Evidence for the existence of monoamine neurons in the cent al nervou system. IV Distribution of monoamine nerv termin ls in the central nervous system. *A t physiol. and* 1965. *64* Suppl. 247 39—85.
64 Fuxe K., Evidence for the existence of monoamine neurons i the central nervou system. III. The monoamine nerv terminal. *Z Zellforsch* 1965 *65* 573—596.
65. F xe, K. and N E. Andén, Studies on central monoamine neurons with special reference to the igro-neostriatal dopamine neuron system. *I* Biochemistry and pharmacology of the basal ganglia Eds. E. Costa, L. J Coté and M. D Yah Ra en Press, Hewlett, N Y 1966, pp. 123—129
66. Fuxe K., A. Dahlström and N A. Hillarp Central monoamine neurons and monoamine neuro-transmission. Proc. of the XXIIIrd Internat. Congr of Physio- logical Sciences, Tokyo Sept. 1965 pp. 419—434
67 Fuxe, K B Hamberger and T Hökfelt, Distribution of noradrenaline nerve termi- nals in cortical areas of the rat. *Brain Res* 1968. *8* 125—131
68. Fuxe, K and T Hökfelt, Further evidence for the existence of tubero-i f ndibular dopamine neurons. *Acta physiol. cand* 1966 *66* 245—246
69 Fuxe K. and T Hökfelt, Monoamine afferent input to the hypothalamus and th dopamine afferent input to the median eminence. *I* Progress in Endo- crinology" Ed. C. Gual. Excerpta Medica I ternat. Congr Series No. 184 1968
70 Fuxe, K. and T Hökfelt, Catecholamines in the hypothalamus and th pituitary gland *I* "Frontiers in Neuroendocrinology" Eds.: W F Ganong nd L Martini. Oxford University Press, Inc. 1969 pp 47—96.

71 Fuxe K. and T Hökfelt, Participation of central monoamine neurons in the regulation of anterior pituitary function with special regard to the neuro-endocrine role of tubero-infundibular dopamin neurons. *In* Aspects of Neuroendocrinology Eds.: W Bargmann and B. Scharrer Springer Verlag, Berlin—Heidelberg—New York, 1970 pp 192—205

72. Fuxe K., T Hökfelt, G Jonsson and U Ungerstedt, Fluorescence microscopy in neuroanatomy *In* "Contemporary Research Methods in Neuroanatomy" Eds. W J H. Nauta and S. O E. Ebbeson, Springer Verlag Berlin—Heidelberg—New York, 1970 pp 275—314

73 Fuxe, K., T Hökfelt and O Nilsson, Observations on the cellular localization of dopamine in the caudate nucleus of the rat. *Z. Zellforsch.* 1964 *63* 701—706

74 Fuxe K. T Hökfelt and O Nilsson, A florescence and electronmicroscopic study on certain brain regions rich in monoamine terminals. *Amer J Anat* 1965 *117* 33—46

75 Fuxe, K., T Hökfelt, O Nilsson and S. Reinius, A fluorescence and electron microscopic study on central monoamine nerve cells. *Anat. Rec* 1966 *155* 33—40.

76 Fuxe, K., T Hökfelt and U Ungerstedt, Localization of indolealkylamines in CNS. *Adv Ph rmacol.* 1968. *6* 235—251

77 Fuxe, K., T Hökfelt and U Ungerstedt, Distribution of monoamines in the mammalian central nervous system by histochemical studies. *I* Metabolism of Amines in the brain Ed. E. Hooper Mac Millan, London. 1969 pp. 10—22

78 Fuxe, K., T Hökfelt and U Ungerstedt, Central monoaminergic tracts. *Prins Psych pharmacol.* 1970. *6* 87—96.

79 Fuxe, K., T Hökfelt and U Ungerstedt, Morphological and functional aspects of central monoamine neurons. *I t. Rev Neurobiol.* 1970 *13* 93—126

80 Fuxe, K., T Hökfelt, U Ungerstedt, N.-E. Andén, L. Butcher and A. Rubensson, The effects of drugs and stereotaxic lesions on the nigro-neostriatal dopamine neurons. *I* "Third Symposium on Parkinson disease Eds F J Gillingham and I. M. Donaldson. E. & S Livingstone Edinburgh and London, 1969 pp. 41—47

81 Fuxe K. and L. Ljunggren, Cellular localization of monoamines in the upper brain stem of the pigeon. *J omp Neu ol.* 1965 *125* 355—382.

82. Fuxe K. and Ch. Owman, Cellular localization of monoamines in the area postrema of certai mammals. *J omp Neurol.* 1965 *125* 337—354

83 Golstein, M., B. Anagnoste A. F Battista, W S Owen and S. Nakatani, Studies of amines in the triatum in monkeys with nigral lesions The disposition, biosynthesis and metabolites of [^{3}H] dopamine and [^{14}C] serotonin in the striatum. *J Neu ochem* 1969 *16* 645—653

84 Goldstein, M., B. Anagnoste W S. Owen and A. F Battista, The effects of ventromedial tegmental lesions on the biosynthesis of catecholamines in the triatum. *Lif Sci.* 1966 *5* 2171—2176.

85 Goldstein M., B. Anagnoste W S. Owen and A. F Battista, The effects of ventromedial tegmental lesions on the disposition of dopamine in the caudate nucleus of the monkey *Brai Res* 1967 *4* 298—300.

86. Harvey J A A. Heller and R. Y Moore, The effect of unilateral and bilateral medial forebrain bundle lesions on brain serotonin *J Pharm col p Ther* 1963 *140* 103—110.

87 Hassler R., Zur Pathologie der Paralysis agitans und des postencephalitischen

Parkinsonismus. *J Psychol N rol.* 1958 *48* 387—476

88. Hassler R., The pathological and pathophysiological basis of tremor and pa kinsonism. Proc 2nd Int. Congr Neuropathol. London 1955 *1* 29—58.

89 Heller A., J A Harvey and R. Y Moore A demonstration of a fall in brai serotonin following central nervous system lesions in the rat. *Biochem Pharma col.* 196.. *11* 859—866.

90. Heller A. and R Y Moore Effect of centr l ervous sy tem lesions on brain monoamines in the rat. *J Ph rma ol e p Ther* 1965 *150* 1—9

91 H ller A., L. S Seiden W Porcher and R. Y Moore Regional effects of lateral hypothalamic lesions on 5-hydroxytryptophan decarboxylase i the cat brain. *J N urochem* 1966. *13* 967—974

92. Hillarp N A., K F xe and A Dahlström, Demonstration and mapping of central neurons containing dopamine noradrenaline and 5-hydroxytryptamine and their reactions to psychopharmaca. *Ph rm R* 1966. *18* 727—741

93 Hillarp N.-A., K. F nd A. Dahlström, Central monoamine neurons *I* Mechanism of release of biogenic amines Eds. U S. Euler S. Rosell and B. Uvnäs. Pergamon Press. 1966 pp. 31—37

94 Hornykiewicz, O Die topische Lokalisation nd das Verhalten on Noradrenalin and Dopamin (3-Hydroxytyramin) in der Substantia nigra des normalen und Parkinson-kranken Menschen *W ien Kl n. W schr* 1963 *75* 309—312.

95 Hornykiewicz, O Zu Frage des Verlauf dopaminerger Neurone im Gehirn des Menschen. *W ie Klin. W sch* 1961 *76* 834—835.

96. Hornykiewicz, O H. J Lisch and A. Springer Homovanillic acid in different regions of the human brain Attempt at localizing central dopamin fibres. *Brain Re* 1968. *11* 662—671

97 Hull, C. D G Barnardi and N A. Buchwald I tracellular responses of caudate neurons to brain stem stimulation. *Brain Re* 1970 *22* 163—179

98. Hyyppä M., A histochemical study of the primary catecholamines in the hypothalamic neurons of the rat in relation t the ontogenetic and sexual differentiation. *Z Zellforsch.* 1969 *98* 550—560

99 Hökfelt, T On the ultrastructural localization of noradrenaline in the central nervous system of the rat. *Z Zellfors h.* 1967 *79* 110—117

100. Hökfelt, T The possible ltrastructural identification of tubero-infundibular dopaminecontaining nerve endings in the median eminence of the rat. *Bra Res* 1967 *5* 121—123

101 Hökfelt, T I vitro studies on central and peripheral monoamine neurons at the ultrastructural level. *Z Zellfors h.* 1968. *91* 1—74

102. Hökfelt, T Electron microscopic tudies on peripheral and central monoamine neurons. M. D. Thesis, 1968 Stockholm.

103 Hökfelt, T and K. Fuxe, Cerebellar monoamine nerve terminals, a new type of afferent fibers to the cortex cerebelli. *E p. Bra R s.* 1969 *9* 63—72

104 Hökf lt, T and U Ungerstedt, Electron and fluorescence microscopical studies on the nucleus caudatus putamen of the rat afte unilateral lesions of ascending nigro-neostriatal dopamine neurons. *Acta physiol. scand* 1969 *76* 415—426.

105 Ishii, T and R. L. Friede, Distribution of catecholamine-binding mechanism in rat brain. *Histochemie* 1967 *9* 126—135

106 Jones, B. E., Catecholamine-containing neurons in the brain stem of the cat and their role in waking Thesis submitted to the university f Delaware, June 1969

Dopamines (= 3 Hydroxytyramin) im Nucleus caudatus nach elektrischer Koagulation des Globus pallidus. Naturwissenschaften. 1964 51 314
144 Sheard, M H. and G K. Aghajanian, Stimulation of midbrain raphé neurons Behavioral effects of serotonin release. Life Sci. 1968 7 19—25
145 Sheard, M. H. and G K. Aghajanian, Stimulation of the midbrain raphé Effect on serotonin metabolism. J Pharm col. p Ther 1968. 163 425—430
146. Sheard, M H., J B. Appel and D X. Freedman, The effect of central nervous system lesions on brain monoamines and behavior J Psychiat. Res 1967 5 237—242.
147 Singh P L. J Poirier and R. Boucher Effect of monoamine oxidase inhibitors on the concentrations f dopamine and serotonin in the striatum of the cat with and without unilateral brain stem lesions. Canad. J Physiol. Pharmacol. 1967 45 897—904
148. Sourkes, T L., Effect of brain tem lesions on the concentration of catecholamines in basal ganglia of the monkey J Neurone g 1966 24 194—195
149 Twarog B. and I H. Page, Serotonin content of some mammalian tissues and urine and a method for its determination. Am. J Physiol. 1953 175 157—161
150 Ungerstedt, U 6-Hydroxydopamine induced degeneration of central monoamine neurons. E p. J Pharmacol. 1968. 5 107—110
151 Ungerstedt, U Histochemical studies on the effects of intracerebral and intraventricular injections of 6-hydroxydopamine on monoamine neurons in the rat brain. I 6-hydroxydopamine and catecholamine neurons. Eds. T Malmfors and H. Thoenen, North-Holland, 1971 101—127
152. Ungerstedt, U Noradrenaline pathways in the rat brain Principal rchitecture. Scie 1971 In press.
153 Ungerstedt, U L. L. Butcher S. G Butcher N E. Andén and K. Fuxe, Direct chemical stimulation of dopaminergic mechanisms in the neostriatum of the rat. Bra Res 1969 14 461—471
154 Vogt, M., The concentration of sympathin in different parts of the central nervous system under normal conditions and after the administration of drugs J Physiol. 1954 123 451—481
155 Vogt, M., Release from brain tissue of compounds with possible transmitter function Interaction of drugs with these substances. Brit. J Pharmacol. 1969 37 325—337
156 York, D H., Possible dopaminergic pathway from ubsta tia nigra to putamen. Brain Re 1969 20 233—249
157 Zeman W and J R. M. Innes, Craigie s Neuroanatomy of the Rat. Academic Press, New York and London 1963

From the Department of Histology Karolinska Institutet, Stockholm 60
Sweden

Striatal Dopamine Release after Amphetamine or Nerve Degeneration Revealed by Rotational Behaviour

By

U Ungerstedt

Abstract

Amphetamine induced vigorous rotational behaviour in rats where the nigro-striatal dopamine (DA) system was unilaterally degenerated by an intra cerebral injection of 6-hydroxydopamine (6-OH DA) The rotational behav iour was analysed in a specially designed "rotometer" and found highly re producable. The intensity of the rotation was proportional to the extent of degeneration in the DA system. The amphetamine site of action was in all probability presynaptic as amphetamine caused a rotation in the opposite direction as compared to the DA receptor stimulating drug apomorphine. Tyrosine hydroxylase inhibition abolished the amphetamine effect, while reserpine potentiated the effect. Evidence was also obtained that the amphetamine induced release of DA was dependent upon nerve impulses. The rotational behaviour reflected the degree of DA receptor stimulation, but changes in the noradrenaline (NA) transmission seemed to modulate the be haviour Decreased NA transmission after dopamine-β-hydroxylase inhibition increased the rotational behaviour The unilateral degeneration of the DA system was studied by the changes in posture and movements that occurred during the degeneration. After inhibition of the monoamine oxidase the rats showed a strong rotation beginning on the 23d and ending on the 33d hour after the operation. The rotational behaviour was probably due to degenera tion release of DA and equivalent to the degeneration contraction of the nictitating membrane in the peripheral nervous system Amphetamine greatly potentiated the spontaneous degeneration release during this period.

Introduction

In a recent article Ungerstedt and Arbuthnott (1970) described a method for functional studies on the nigro-striatal dopamine (DA) system in rats. Unilateral degeneration of the DA system by an intracerebral injection of 6-hydroxydopamine (6-OH DA) and subsequent treatment with amphetamine, induced a pronounced rotational behaviour which was quantified in a specially designed rotometer Because of its dose dependency and reproducability the response was thought to reflect the amphetamine induced DA release (Glowinski and Axelrod 1965 Carlsson *et al* 1966) This study further analyses the specificity and the reproducability of the amphetamine induced rotational behaviour The method is applied to the problems of amphetamine site of action, interaction with reserpine and relative importance of noradrenaline (NA) and DA neurons in eliciting the rotational behaviour

The functional changes associated with degeneration of the peripheral NA neurons have been carefully studied (Trendelenburg 1966) The corresponding events in the central nervous system have so far been experimentally inaccessible. However the rotational method permits a continuous analysis of the degeneration process in the nigro-striatal DA system. In this paper it has been possible to reveal changes comparable to the degeneration contraction of the nictitating membrane. The process is profoundly influenced by nialamide and amphetamine.

Material and Methods

The experiments were performed on 104 male Sprague Dawley rats with a body weight of 150 ± 5 g at the time of the stereotaxic operation.

Stereotaxic operation

The animals were operated in a David Kopf stereotaxic instrument during anaethesia maintained by a mixture of oxygen, nitrous oxide and Halothane which was circulated through a mask fitted over the nose of the animal. *Electrocoagulations* were performed with a 0.25 mm stainless steel needle insulated except for 0.4 mm at the tip The indifferent lead was inserted into the rectum. High frequency current was generated by a Siemens Radiotom. Electrocoagulations were performed unilaterally in the bundle of DA axons rostral to the area entralis tegmenti. This lesion interrupts the nigro-striatal DA system as was demonstrated by fluorescence histochemistry and electron-microscopy (Hökfelt and Ungerstedt 1969) *Intracerebral injections of 6-hy-*

droxydopamine were made through a Hamilton cannula 0.2 mm in diameter. The fluid was delivered by a motor driven "Agla micrometer all glass syringe" (Burroughs and Wellcome Co England) at a speed of 1 μl per minute. 6-hydroxydopamine dihydrobromide (Regis) (6-OH DA) was dissolved in saline and ascorbic acid (0.2 mg/ml) was added to prevent auto-oxidation. 8 μg of 6-OH DA (calculated as the base) was injected in a volume of 4 μl. The injections were performed unilaterally into the rostral part of the DA cell group in the substantia nigra with the stream of fluid directed caudally. Injections were also performed unilaterally into the caudal portion of the cell group as well as clearly behind the cells. For further details on the stereotaxic procedures, see Ungerstedt (1971a)

Recording of rotational behaviour

A number of drugs (see below) were tested for their ability to induce or modify rotational behaviour in the operated animals. The rotational behaviour was recorded by placing the rat in a rotometer (Ungerstedt and Arbuthnott 1970) consisting of a bowl, shaped as a half sphere (fig 1) The rat was connected to the geometrical center of the sphere by a thin wire. The wire extended from a harness fitted over the chest of the animal to a microswitch arrangement in the center of the sphere that reacted to each full turn of the animal. Because of the spherically shaped surface the animal was able to move around comfortably without pulling the wire. The rotational behaviour was registered on electromechanical counters and plotted either as turns per minute versus time or total number of turns made under the influence of a certain drug In order to visualise in which direction the rat was rotating rotational behaviour in the direction towards the lesioned side was plotted with positive y-values, whereas rotational behaviour towards the non-lesioned side was plotted with negative y values (for details of the method, see Ungerstedt and Arbuthnott 1970) (fig 1)

Histochemistry

After the pharmacological testing (see below) representative animals (about 2/3 of all animals) were killed and analysed with the Falck and Hillarp histochemical technique for biogenic monoamines (Falck 1962 Falck *et al.* 1962 Corrodi and Jonsson 1967 Fuxe *et al.* 1970) The animals were decapitated during light chloroforme anaesthesia, the brains quickly removed, cut in four frontal sections, placed on stiff pieces of paper with a nylon net on top of the tissue piece and then frozen in propane cooled by liquid nitrogen. The brain

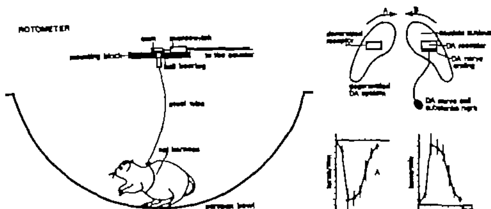


Fig. 1 *Left* Schematic drawing of the otometer The movements of the rat are transferred by the steel wire to the microswitch arrangement.

Upper right Principal outline of the experimental situation shown in a horizontal projection of the nigro-striatal DA system. When the stimulation of the denervated receptor dominates, the animals rotates in direction A. When stimulation of the innervated receptor dominates the animal rotates in direction B.

Lower right The rotational behaviour is presented as turns per min ersus time. The curves are given negative y-values when stimulation of the denervated receptor dominates and positive y-values when stimulation of the innervated receptor dominates.
Each point represents the mean ± S.E.M. of certain number of animals.

pieces were freeze-dried for 2 1/2—3 1/2 days in a FT 1 freeze drier (Bergman and Beving AB, Stockholm) reacted with gaseous formaldehyde embedded in liquid paraffine, sectioned, mounted and studied in a fluorescence microscope (for details on the histochemical procedures and the freeze-drier see Fuxe *et al* 1970 Olson and Ungerstedt 1970)

Spontaneous behaviour

Animals with unilateral lesions of the nigro-striatal DA system were observed over a period of two years after the operation. Spontaneous rotational behaviour and postural asymmetries were studied when the animals were left undisturbed in their cages and when they were removed and disturbed by handling, pinching of their tails, sudden sounds and so on.

Drug induced behaviour

The effect of a number of drugs were tested in the rotometer after unilateral lesions of the nigro-striatal DA system. All drugs were injected *i p* The num-

ber of animals tested are indicated within brackets. The following drugs were used

dl-amphetamine sulfate, nialamide (Swedish Pfizer Näsbypark) spiroperidol (Janssen Beerse) haloperidol (Leo, Helsingborg) FLA-63 (Astra, Södertälje) reserpine (Swedish Ciba, Stockholm) and DL-α-methyltyrosine methylester HCl (H44/68 Hässle Göteborg) The doses given refer to the form indicated above except for amphetamine that was calculated as the base.

1 *Specificity and reproducibility of the response to amphetamine*

a. Animals were treated with amphetamine (5 mg/kg) 7 days after the injection of 6-OH DA into the caudal portion of the substantia nigra (5 animals) and 7 days after an injection clearly behind the DA cell bodies (5 animals)

b. Two months after the injection of 6-OH DA into the substantia nigra animals were treated with three doses of amphetamine (5 mg/kg) two days apart *i.e* on day 1 3 and 6 (5 animals)

2. *Drug interference with the response to amphetamine*

A number of drugs were tested for their effect on the response to amphetamine. Animals were injected with 6-OH DA into the substantia nigra and tested in the rotometer 1—4 months later·

a. Amphetamine (1 mg/kg) Two days later H44/68 (250 mg/kg) + amphetamine (1 mg/kg) 2 hours later (5 animals)

b Amphetamine (? mg/kg) Six hours later reserpine (10 mg/kg) 48 hours after reserpine, amphetamine (2 mg/kg) (6 animals)

c. Amphetamine (5 mg/kg) Two days later FLA-63 (25 mg/kg) + amphetamine (5 mg/kg) 30 min later (4 animals)

d. Amphetamine (5 mg/kg) + haloperidol (1 mg/kg) or spiroperidol (0 1 mg/kg) 1 hour later (6 animals)

3. *Effect of nialamide and amphetamine during the time of neuronal degeneration*

Nialamid (100 mg/kg) was given directly after the injection of 6-OH DA into the substantia nigra (8 animals) The animals were immediately placed in the rotometer and the rotational behaviour was registered for a period of 48 hours.

Amphetamine (5 mg/kg) was administered to different animals 15 min, 1 2 4 6 12 18 24 30, and 48 hours after injection of 6-OH DA into the substantia nigra (3? animals) Amphetamine was also administered 1 2, 3 6 12, 18 24 30, 42, 48 and 56 hours after electrocoagulation of the ascending

DA axons (18 animals). Six animals received repeated injections of amphetamine (2 mg/kg) during the course of the degeneration, i.e. 12, 24, 36, 48, 60 and 72 hours after the 6-OH DA injection into the substantia nigra.

Results

Histochemistry

The fluorescent histochemical analysis showed that the substantia nigra was devoid of fluorescence DA cell bodies except in a few animals where a couple of cells remained in the periphery of the nucleus. The site of injection was usually revealed by an area of necrosis about 0.3—0.5 mm in diameter in the center of the substantia nigra. The DA nerve terminals had disappeared from the ipsilateral corpus striatum and there was a varying disappearance of DA nerve terminals also from the nucleus accumbens. After electrocoagulation of the ascending DA axons there was a more pronounced disappearance of the DA nerve terminals from the nucleus accumbens and the tuberculum olfactorium. After the injection of 6-OH DA into the caudal part of the substantia nigra a number of DA cells remained in the rostral part of the nucleus and there was only a partial denervation of the corpus striatum. However, when the 6-OH DA injection was performed well behind the substantia nigra there was no damage to the DA cell bodies and the corpus striatum exhibited a normal fluorescence. The density of NA nerve terminals in the ipsilateral hypothalamus were slightly lowered after all 6-OH DA injections regardless if they were within or behind the substantia nigra. The electrocoagulations produced about the same degeneration of NA nerve terminals in the hypothalamus as did the 6-OH DA injections.

Spontaneous behaviour

The animals woke up 10—15 min after the injection of 6-OH DA into the substantia nigra. The excitation at the moment of waking up causes a normal animal to run and stumble forward. The operated animals, however, engaged in a rapid rotation towards the lesioned side. This behaviour ceased if the animal was left undisturbed, but was resumed if the animal was irritated, e.g. by pinching of its tail. When the rotational behaviour ceased they assumed an asymmetrical posture. The limbs on the side ipsilateral to the lesion were kept close to the body while the contralateral limbs were extended. The head and the tail deviated towards the side of the lesion (fig. 2). When the animal was left alone in its cage it moved around in circles towards the operated side. One day after the operation the rotational behaviour was even more pronounced



Fig Typical posture after 6-OH-DA induced chronic degeneration of the left nigro-striatal DA system. The body deviates toward the side of the lesion.

when the animals was disturbed, e.g by knocking on the cage, clapping of hands, pinching its tail or dropping it on the floor However several animals showed a slow rotation towards the non-lesioned side when left absolutely undisturbed in their cages. They also exhibited a continuous weak sniffing behaviour Two days after the 6-OH DA injection the slow spontaneous rotation and the sniffing behaviour was not observed, only the deviation towards the operated side in movements and posture remained The tendency to turn towards the lesioned side during normal activities in the cage soon ceased and the asymmetry became obvious only when the animals were disturbed Even after two years the animals would turn or rotate towards the operated side when pinching their tails.

Three weeks after the 6-OH DA injection another type of behaviour was noted When the animals were placed in the rotometers they suddenly engaged in an explosive rotational behaviour towards the unoperated side making 30—50 turns per minute during a period of 1—2 minutes. The behaviour then ceased and they resumed their asymmetrical posture towards the operated side. The behaviour seemed connected to the stress of handling and the new environment. It usually did not occur if the animals were tested repeatedly Animals showing this type of behaviour regularly showed a strong rotational behaviour after amphetamine (see below) The behaviour was noted 1 3 4 5 7 and 8 months after the operation and showed no tendency to disappear

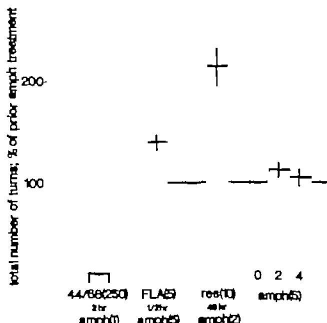


Fig. 3 Effects of various drugs on the amphetamine induced rotation in animals unilaterally denervated with 6-OH DA. The total number of turns are expressed as per cent of a treatment with amphetamine two days earlier Each volumn represents th mean ± S.E.M. for 4—6 animals. The following drugs are abbreviated H44/68 FLA63 reserpine and amphetamine. Doses in mg/kg are indicated within brackets. The time intervals between the administration of the different drugs are indicated. The three values for amphetamine only represent reproducability test f the rotation on day 2 and 4 as compared to day 0.

Drug induced behaviour

1 *Specificity and reproducability of the response to amphetamine*

a. The histochemical analysis showed that injection of 6-OH DA into the caudal portion of the substantia nigra produced a partial denervation of the corpus striatum while 6-OH DA injection behind the DA cell group did not affect the nigro-striatal DA system. The extent of denervation was reflected in the rotational behaviour Seven days after a 6-OH DA injection into the caudal part of the substantia nigra amphetamine induced rotation towards the operated side This rotation, however was weaker than that occurring after an injection of 6-OH DA that reached all DA cell bodies and thus produced a more extensive degeneration. Amphetamine did not induce any rotation in animals where 6-OH DA did not affect the nigro-striatal DA system.

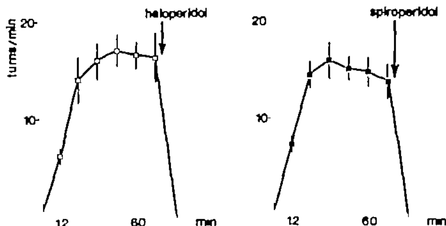


Fig. 4 Inhibition of the amphetamine induced rotation by haloperiodol and spiroperidol dministered during the course of the rotational behaviour

b When the response to amphetamine was tested in chronic animals by treatment every second day there was a reproducable response to the drug (fig 3)

2 Drug interference with the response to amphetamine

a. Pretreatment with the tyrosine hydroxylase inhibitor H44/68 (250 mg/kg) strongly inhibited the response to amphetamine (fig 3)

b. Pretreatment with reserpine (10 mg/kg) strongly increased the response to amphetamine 48 hours after reserpine The response showed a 100 % in crease (fig 3)

c. Pretreatment with FLA-63 (25 mg/kg) increased the rotation after am phetamine (fig 3)

d Haloperidol (1 mg/kg) and spiroperidol (0 1 mg/kg) stopped the rotation after amphetamine within 3 minutes (fig 4)

3 Effect of nialamide and amphetamine during the time of neuronal d generation

The administration of nialamide directly after the operation did not change the usual postoperative behaviour of the animals, *i.e* they still showed a strong tendency to turn towards the operated side Around 20 hours after the 6-OH DA injection or electrocoagulation the animals developed a restless behaviour

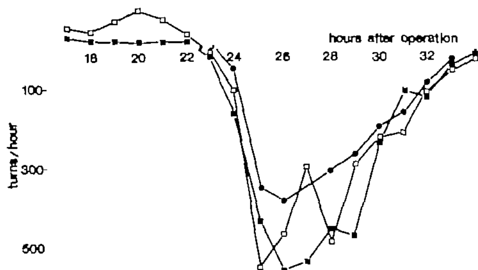


Fig 5. Rotational behaviour during unilateral degeneration of the nigro-striatal DA system after 6-OH-DA injection into the substantia nigra. Nialamid (100 mg/kg) was given directly after the operation. Each curve shows the number of turns per hour made by one representative animal. Positive y-values indicate a dominance of the intact DA system, while negative y-values indicate dominance of the lesioned DA system (see fig. 1)

of moving and sniffing Around 23 hours after the operation the animals started to rotate toward the unoperated side (fig 5) This rotation continued for 8—10 hours. About 36 hours after the operation the animals were again asymmetrical towards the operated side.

Amphetamine induced three different types of rotational behaviour during th period of nerve degeneration after the lesion of the nigro-striatal DA system with 6-OH DA or electrocoagulation. The behaviour was the same if amphetamine (5 mg/kg) was given at different time points to different animals or if amphetamine (2 mg/kg) was given repeatedly to the same animal. 1 The animals rotated only towards the operated side. 2 The animals rotated towards the unoperated side initially and then changed direction and rotated towards the operated side 3 The animals rotated only towards the unoperated side. These behaviours altered as a function of the time after the operation. Table 1 and 2 show the number of animals exhibiting these different types of behaviour at various time points after the 6-OH DA injection or electrocoagulation.

TABLE 1. Effect of amphetamine (5 mg/kg) during degeneration of the DA neurons after unilateral 6-OH DA injection into the substantia nigra. Left: operated side right: unoperated side.

Hrs after operation	Rotational behaviour		
	Only left rotation no. of animals	Shift from right to left no. of animals	Only right rotation no. of animals
1/2	1	2	
1	1	4	
2—30			18
48	5	1	

TABLE 2. Effect of amphetamine (5 mg/kg) during degeneration of the DA neurons after unilateral electrocoagulation of the DA pathway. Left: operated side right: unoperated side.

Hrs after operation	Rotational behaviour		
	Only left rotation no. of animals	Shift from right to left no. of animals	Only right rotation no. of animals
1—3	4		
6—18		5	
24—30			2
42—48		5	
56	4		

Figure 6 shows the actual rotational behaviour of one representative animal at different time points after the 6-OH DA injection. The rotational behaviour after electrocoagulation was very similar to that after 6-OH DA with the only difference that the events occurred sooner after 6-OH DA lesions. Already 15 min after the 6-OH DA injection there was a short period of rotation towards the unoperated side while this occurred 5—6 hours after an electrocoagulation. The final rotation towards the operated side remained for both categories of operated animals. The longest period studied was two years.

Discussion

Degeneration of the nigro-striatal DA system is associated with disturbance of motor function both clinically and experimentally. By damaging the DA

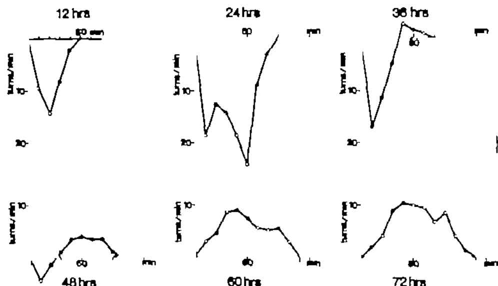


Fig 6. Rotational behaviour induced by amphetamine (2 mg/kg) at various time points during the 6-OH DA induced degeneration of the nigro-striatal DA system in one representative animal. Positive y-values indicate a dominance of the intact DA system, while negative y values indicate a dominance of the lesioned DA system (see fig 1)

pathway or the corpus striatum unilaterally it is possible to create an experimental situation where pharmacologically induced DA release occurs only on one side In rats this imbalance is revealed as a vigorous rotational behaviour towards the lesioned side after treatment with a monoamine oxidase inhibitor + reserpine (Andén *et al* 1966) or with amphetamine (Andén *et al* 1967b) Imbalance in the DA content of the corpora striata can also be induced by direct unilateral injection of DA into the striatum after pretreatment with nialamide This induces a rotational behaviour towards the side contralateral to the DA injection (Ungerstedt *et al* 1969) There is, thus, considerable support for the conclusion that a unilateral increase in the DA transmission causes the rat to move towards the side where less DA is available.

It seemed reasonable to assume that the degree of motor asymmetry reflected the difference in DA receptor stimulation between the two striatum. The method of intracerebral injection of 6-OH DA (Ungerstedt 1968 1971b) made possible a relatively selective and reproducable removal of the DA system unilaterally and created the prerequisite for a quantitation of the rotational behaviour We therefore constructed a "rotometer where the animal was able to move around freely within a limited area while its rotational speed was recorded in turn per minute (Ungerstedt and Arbuthnott 1970) Amphet

amine was found to induce a reproducable and dose dependent rotational behaviour which supported the theory that the rotational behaviour was related to the degree of DA receptor stimulation (Ungerstedt and Arbuthnott 1970) Apomorphine, which stimulates DA receptors (Andén *et al* 1967b Ernst 1967) induced a rotation in the opposite direction as compared to amphetamine Hence, the denervated striatum was more sensitive to apomorphine than the innervated which was interpreted as a postsynaptic supersensitivity occurring as a result of the denervation (Ungerstedt 1971a) It is, thus, possible to tell a drug that stimulates DA receptors from a drug that increases DA release by the direction of the rotation.

The purpose of this article is to present some results obtained with the "6-OH DA rotation model" in relation to the site of action of amphetamine, its interaction with reserpine and the relative role of NA and DA neurons in the response to the drug The effect of amphetamine is also studied in connection with acute lesions of the DA pathway which reveals a relationship between nerve impulses and amphetamine action as well as some features of nerve degeneration. The experiments performed with the "6-OH DA rotation model" are based upon the histochemical (Ungerstedt 1968, 1971b) and electronmicroscopic (Hökfelt and Ungerstedt 1971) evidence showing the selectivity of the neuronal damage caused by 6-OH DA injections as compared to conventional lesion methods. However there is always some unspecific damage that has to be accounted for In order to exclude that damage to the ascending NA axons or to unknown ascending pathways might be responsible for the motor asymmetries, animals were injected with 6-OH DA in such a way that all DA cells in the substantia nigra, part of the cells or no cells degenerated Amphetamine induced a rotation which was related to the number of degenerated cells When 6-OH DA was injected behind the substantia nigra there was no rotation, when injected into the caudal half there was a weak rotation and when involving the whole nucleus there was a strong rotation. This seems like a good indication that the rotational behaviour is indeed an expression of an imbalance in the DA transmission of the two corpus striatum. Apart from the specificity of the response it is obviously important that the experimental animals may serve as their own controls. Hence, amphetamine was administered repeatedly and the response was found reproducable when amphetamine (5 mg/kg) was administered for three times with two days intervals (fig 3)

Amphetamine was originally thought to act on the central nervous system by inhibiting monoamine oxidase (MAO) (Mann and Quastel 1940) However when it was found that the action of amphetamine persisted after depletion of the monoamine stores by reserpine it was proposed that amphetamine

stimulated 5-hydroxytryptamine (5-HT) receptors (Vane 1960) or catechol amine receptors (van Rossum *et al* 1962 Smith 1963) Stein (1964) suggested on the basis of self stimulation experiments that amphetamine had an indirect mode of action and the subsequent development of specific inhibitors of tyrosine hydroxylase revealed that the action of amphetamine was dependent upon an intact transmitter synthesis indicating a presynaptic point of action (Weissman *et al* 1966 Randrup and Munkvad 1966 Hansson 1966) Biochemical and histochemical work demonstrated that amphetamine did release catecholamines from central NA and DA neurons (Glowinsky and Axelrod 1965 Carlsson *et al* 1966 Fuxe and Ungerstedt 1968) The present results with the 6-OH DA rotation model give further support for the conclusions above Pretreatment with the synthesis inhibitor H44/68 inhibited the amphetamine induced rotation (fig 3) and the fact that amphetamine treated animals rotated in the opposite direction compared to animals treated with the DA receptor stimulating drug apomorphine (Andén *et al.* 1967b Ernst 1967 Ungerstedt 1971a) shows that the action of amphetamine is mainly presynaptic (see above)

In spite of the above evidence the question remains, why depletion of the monoamine stores with reserpine does not inhibit the action of amphetamine (van Rossum *et al.* 1962 Smith 1963 Rech 1964) However the fact that DA synthesis continues after reserpine (Andén *et al.* 1964) makes possible a release of DA from the amphetamine sensitive, extragranular (Carlsson *et al* 1966) storage sites. The recent description of postsynaptic supersensitivity after reserpine (Ungerstedt 1971a) provides an additional explanation to the fact that reserpine actually enhances the effect of amphetamine. In the present investigation there was a 100 % increase in the response to amphetamine 48 hours after reserpine.

The rotational behaviour after amphetamine is obviously dependent upon the unilateral release of DA. However the concomittant release of NA may influence the rotational behaviour In order to evaluate the relative importance of DA and NA release animals were pretreated with the DA-hydroxylase inhibitor FLA-63 (Corrodi *et al.* 1970) which significantly increased the response to amphetamine (fig 3) Haloperidol which blocks NA and DA receptors, and spiroperidol, which mainly blocks DA receptors (Andén *et al.* 1967a, 1970) both inhibited the rotation (fig 4) The results indicate that stimulation of DA receptors induces the rotation while changes in the NA transmission at the most modulate the rotational behaviour The surprising increase in the response to amphetamine after a decrease in the NA transmission may seem contradictory to the evidence that DA release mainly induces stereotyped behaviour while NA release is involved in other forms of be

haviour such as locomotion (Sheel-Krüger and Randrup 1967) However the locomotion involved in the rotational behaviour may well be regarded as highly stereotyped It is probably comparable to the stereotyped forward walking, or obstinate progression, observed after bilateral injection of amphetamine into the striatum of reserpine treated rats (Fuxe and Ungerstedt 1970)

Inhibition of the NA transmission with FLA63 seems to decrease the exploratory component of the behaviour and increase the stereotyped behaviour in an open field situation (Fuxe and Ungerstedt 1970) This probably corresponds to the increased rotational behaviour found in the present experimental situation.

Apart from the pharmacologically induced release of DA by *e.g* amphetamine there is another kind of DA release during the neuronal degeneration after a lesion. This type of release reflects the mechanisms of uptake, synthesis and storage of the transmitter as well as the endurance of the cellular components. We have studied the degeneration release after unilatral lesions of the nigro-striatal DA system in three different ways The spontaneous rotational behaviour occurring after the lesion. The rotational behaviour occurring after lesions when MAO is inhibited and finally the effect of amphetamine on the degenerating DA neurons.

The asymmetry after a lesion is apparent as soon as the animal wakes up from the anaesthesia. This is in agreement with the increased retention of striatally injected ³H DA found after such a lesion in a previous study (Ungerstedt 1971b) and indicates a decrease in nerve impulse activity on the lesioned side. The spontaneous postural asymmetry as well as the rotational behaviour induced by activation of the animal shows that the animal is initially unable to compensate for the lesion. The slow rotational behaviour towards the unoperated side in the undisturbed animals about 24 hours after the lesion is a sign of increased DA activity on the lesioned side probably due to degeneration release. The chronic animal soon compensates for the unilateral denervation during its normal activities and it is only revealed when the animal is disturbed. About a month after the denervation the peculiar explosive rotation towards the unoperated side may be induced in stressful situations. The direction of this rotation indicates that the denervated side is activated in a dopaminergic way" However it seems to occur in those animals which have the most extensive denervation of the striatum and show the strongest rotational behaviour after DA releasing drugs as well as DA receptor stimulating drugs. There is no obvious explanation to the phenomenon except that the striatal cells are supersensitive to dopaminergic influence" and may possibly react in that direction on a number of stimuli.

In order to visualise the degeneration release of the transmitter the animals were treated with the MAO inhibitor nialamide directly after the 6-OH DA lesion of the nigro-striatal DA system. The DA leaking out from degenerating storage sites was protected from being broken down and thus able to reach the DA receptor where its presence was revealed by the rotational behaviour. The degeneration release was dramatic and occurred suddenly between the 23d and the 33d hour after the operation (fig. 5).

The time course of the degeneration release is in agreement with electron-microscopical and histochemical studies on the loss of DA from the degenerating DA nerve terminals after 6-OH DA injection into the substantia nigra (Hökfelt and Ungerstedt 1971, Ungerstedt 1971b).

The functional changes during degeneration have been studied extensively in the peripheral nervous system, especially after removal of the superior cervical ganglion (Trendelenburg 1966). In the central nervous system, it has not so far been possible to find a functional variable that reflects the changes in the monoamine transmission. The 6-OH DA rotation model, however, permits a quantitative analysis of the changes in the nigro-striatal DA system and it is of obvious interest to compare the degeneration of the central and the peripheral monoamine neurons.

The slow spontaneous rotation seen around 24 hours after the 6-OH DA lesion is probably analogous to the well known degeneration contraction of the nictitating membrane (Langer 1966). However, the weak spontaneous rotation compared to the pronounced rotation after treatment with nialamide indicates that the DA leaking out from the degenerating nerve endings is efficiently broken down by MAO. It is obviously difficult to make a correct comparison with the degeneration of the peripheral sympathetic nerves. However, Lundberg (1970) has studied the degeneration contraction of the periorbital smooth muscle after an identical MAO inhibition. The duration of the degeneration phenomena are practically identical in the two systems, i.e. about 10 hours. The onset, however, differs considerably. The peripheral degeneration contraction starts after 14 hours, while the central starts after 23 hours.

The lesioned nigro-striatal pathway is an interesting preparation for studies of the amphetamine induced release of DA. During the period shortly after the lesion the system is virtually intact except for the lack of nerve impulses. As the degeneration progresses it is reasonable to expect a change in the intraneuronal storage of the transmitter which might be revealed by the response to amphetamine (table 1 and 2). When amphetamine was administered 30 min—3 hours after an electrocoagulation of the ascending DA axons the animals rotated towards the lesioned side indicating that more DA was released from the intact side. Six hours after the electrocoagulation of the DA

axons the animals started with a short period of rotation towards the non lesioned side and then changed direction. Hence, there was initially a short period when the DA release on the lesioned side dominated At 24 hours the animals rotated only towards the non lesioned side upon amphetamine treatment, demonstrating a total dominance of the release from the degenerating DA nerve terminals. After 56 hours the animals rotated only towards the lesioned side *i.e.* the non lesioned side dominated. The events were essentially the same when amphetamine was administered after 6-OH DA lesions (table 1 and fig 6) except that the whole process seemed to be somewhat speeded up which is in agreement with the fact that the nigro-striatal DA system degenerates somewhat faster after 6-OH DA lesions as compared to electro-coagulations (Ungerstedt 1971b)

The initial dominance of the non-lesioned side is very interesting as the nerve impulses are lacking on the lesioned side. The results indicate that the amphetamine induced release of DA is dependent upon nerve impulse activity The increasing dominance of the lesioned side that starts between 3 and 6 hours after the lesion may be explained by the recent finding that the amount of DA in the striatum is more than doubled 3 hours after a lesion of the axons probably because the DA synthesis continues in spite of the lack of nerve impulses (Andén *et al* 1971 and to be published) This greatly increased amount of DA is either localised in an amphetamine sensitive pool or the increased release after amphetamine treatment may be due to the beginning of a break down of the intraneuronal storage sites. However the fact that the degeneration release of DA, as detected after MAO inhibition, occurred between 23 and 35 hours after the lesion indicates that the increased release after amphetamine initially is due to the increased amount of DA in the neuron

The extensive dominance of the rotation induced from the lesioned side 24 hours after the electrocoagulation is probably depending upon a series of phenomena. There is an increased amount of DA in the nerve terminals (Andén *et al.* 1971 Nybäck, pers. comm.) the degeneration break down of the storage sites has begun and the postsynaptic supersensitivity has started to develop (Ungerstedt 1971a) The final stage is reached when the degenerating neurons have lost their DA and the non-lesioned side dominates, *i.e.* the animals rotate only towards the lesioned side after amphetamine.

The fact that tyramine excerts a potentiating action on the degeneration contraction of the nictitating membrane (Langer and Trendelenburg 1966) is very interesting in comparison to the similar effect excerted by amphetamine in the present study However so far it is not possible to determine if this effect has a granular or an extragranular site of action.

Acknowledgement

This work was supported by grant B71 14X 715-06C from the Swedish Medical Research Council and by grants from Svenska Livförsäkringsbolags Nämnd för Medicinsk Forskning and Ollie and Elof Ericssons Stiftelse. I am greatly indebted to Miss Eva Henriksson and Miss Kerstin Sterner for their excellent technical assistance.

References

Andén N. E., S. G. Butcher, H. Corrodi, K. Fuxe and U. Ungerstedt, Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *European J. Pharmacol.* 1970 *11* 303—314

Andén, N. E., H. Corrodi, K. Fuxe and T. Hökfelt, Increased impulse flow in bulbo-spinal noradrenaline neurons by catecholamine receptor blocking agents. *European J. Pharmacol.* 1967a. *2* 59—64

Andén, N.-E., A. Dahlström, K. Fuxe and K. Larsson, Functional role of the nigro-neostriatal dopamine neurons. *Acta pharmacol. (Kbh.)* 1966. *24* 263—274

Andén N. E., B. E. Roos and B. Werdinius, Effects of chlorpromazine, haloperidol and reserpine on the levels of phenolic acids in rabbit corpus striatum. *Life Sci* 1964 *3* 149—158.

Andén, N.-E., A. Rubenson, K. Fuxe and T. Hökfelt, Evidence for dopamine receptor stimulation by apomorphine. *J. Pharm. Pharmacol.* 1967b. *19* 627—629

Carlsson, A., K. Fuxe, B. Hamberger and M. Lindqvist, Biochemical and histochemical studies on the effects of imipramine-like drugs and (+) amphetamine on central and peripheral catecholamine neurons. *Acta physiol. scand.* 1966. *67* 481—497

Corrodi, H., K. Fuxe, B. Hamberger and A. Ljungdahl, Studies on central and peripheral noradrenaline neurons using a new dopamine-β-hydroxylase inhibitor. *European J. Pharmacol.* 1970 *12* 145—155.

Corrodi, H. and G. Jonsson, The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines. A review on the methodology. *J. Histochem. Cytochem.* 1967 *15* 65—78.

Ernst, A. M., Mode of action of apomorphine and dexamphetamine on gnawing compulsion in rats. *Psychopharmacologia (Berl.)* 1967 *10* 316—323

Falck, B., Observations of the possibilities of the cellular localization of monoamines by fluorescence method. *Acta physiol. scand.* 1962. *56* Suppl. 197 1—26.

Falck, B., N.-A. Hillarp, G. Thieme and A. Torp, Fluorescence of catecholamines and related compounds condensed with formaldehyde. *J. Histochem. Cytochem.* 1962. *10* 348—354

Fuxe, K., T. Hökfelt, G. Jonsson and U. Ungerstedt, Fluorescence microscopy in neuroanatomy. *In* "Contemporary research methods in neuroanatomy". Eds. W. Nauta and S. Ebbesson. Springer 1970 275—314

Fuxe, K. and U. Ungerstedt, Histochemical studies on the effect of (+)-amphetamine drugs of the imipramine group and tryptamine on central catecholamine and 5-hydroxytryptamine neurons after intraventricular injection of catecholamines and 5-hydroxytryptamine. *European J. Pharmacol.* 1968. *4* 135—144

Fuxe, K. and U. Ungerstedt, Histochemical, biochemical and functional studies on central monoamine neurons after acute and chronic amphetamine administration. *In* Amphetamine and related compounds. Eds. E. Costa and S. Garattini. Raven Press, New York 1970. 257—288.

Glowinski, J. and J. Axelrod, Effect of drugs on the uptake, release and metabolism of H^3-norepinephrine in the rat brain. *J. Pharmacol.* 1965. *149* 43—49

Hanson, L. C. F., Evidence that the central action of amphetamine is mediated via catecholamines. *Psychopharmacologia (Berl.)* 1966. *9* 78—80.

Hökfelt, T. and U. Ungerstedt, Electron and fluorescence microscopical studies on the nucleus caudatus putamen of the rat after unilateral lesions of ascending nigro-neostriatal dopamine neurons. *Acta physiol. scand.* 1969 *76* 415—426.

Hökfelt, T and U Ungerstedt, Effects of 6-hydroxydopamin on central monoamine neurons with special reference t the nigro-striatal dopamine system: An electron and fluorescence microscopical study *B ain Res.* 1971 In press.

Langer S. Z., The degeneration contraction of the nictitating membrane in the unanesthetized cat. *J Pharmacol. e p Ther* 1966 *151* 66—72

Langer S. Z. and U Trendelenburg, The onset of denervation supersensitivity *J Pharmacol. xp Ther* 1966 *1 1* 73—86

Lundberg, D The degeneration contraction of a sympath tically innervated smooth muscle in th rat after reserpine, inhibition of monoamine oxidase or tyrosine hydroxylase *Acta physiol. scand* 1970 *80* 107—116

Mann P J G and J H. Quastel, Benzedrine and brain metabolism. *Biochem. J* 1940 *34* 414

Olson, L. and U Ungerstedt, A simple high capacity freeze-drier for histochemical use. *Histochemie* 1970 *22* 8—19

Randrup A. and J Munkvad, Role of catecholamines in the amphetamine excitatory response. *Natu e* 1966. *211* 540

Rech, R. H., Antagonism of reserpine behavioral depression by d-amphetamine *J Pharmacol. exp Ther* 1964 *146* 369—376.

Rossum, Van, J M., J B. van der Schoot and J A. Th. Hurkmans Mechanism of action of cocaine and amphetamine in the brain. *Experientia* 1962. *18* 229—231

Scheel-Krüger J and A. Randrup Stereotype hyperactive behaviour produced by dopamine in the absence of noradrenaline. *Lif Sci.* 1967 6 1389—1398

Smith, C. B., Enhancement by reserpine and α-methyl DOPA of the effects of d-amphetamine upon the locomotor activity of mice. *J Pharmacol. exp Ther* 1963 *142* 343—350

Stein, L. Self-stimulation of the brain and the central stimulant action of amphetamine. *Fed P oc* 1964 *23* 836—850

Trendelenburg, U Mechanisms of supersensitivity and subsensitivity to sympathomimetic amines. *Pharm col. Rev* 1966 *18* 629—640.

Ungerstedt, U 6-Hydroxydopamine induced degeneration of central monoamine neurons. *European J Pharma ol* 1968. *5* 107—110.

Ungerstedt, U Postsynaptic supersensitivity after 6-hydroxydopamine induced degenera tion of the nigro-striatal dopamine system in the rat brain. *Act physiol. scand* 1971a. Suppl. 367 69—93

Ungerstedt, U Histochemical studies on the effects of intracerebral and intraventricular injections of 6-hydroxydopamine on monoamine neurons in the rat brain. *I* "6-Hydroxydopamine and catecholamine neurons" Eds. T Malmfors and H Thoenen. North-Holland 1971b. 101—127

Ungerstedt, U and G Arbuthnott, Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigro-striatal dopamine system. *Brain Res* 1970 *24* 485—493

Ungerstedt, U L. L. Butcher S. G Butcher N E. Andén and K. Fuxe, Direct chemical stimulation of dopaminergic mechanisms in the neostriatum of the rat. *B ain Res* 1969 *14* 461—471

Vane, J R The actions of sympathomimetic amines on tryptamine receptors. *I* Adrenergic mechanisms Eds. J R. Vane G E. W W lstenholme and M. O Connor J A Churchill Ltd. London. 1960. 356.

Weissman, A B K Koe and S Tenen, Antiamphetamine effects following inhibition of tyrosine hyd oxylase *J Pharmacol.   p Ther* 1966. *151* 339—352.

From the Department of Histology Karolinska Institutet, Stockholm
Sweden

Postsynaptic Supersensitivity after 6 Hydroxy-dopamine Induced Degeneration of the Nigro striatal Dopamine System

By

U Ungerstedt

Abstract

The effect of L-DOPA and the dopamine (DA) receptor stimulating drug apomorphine was studied in rats after unilateral degeneration of the nigro-striatal DA system by intracerebral injection of 6-hydroxydopamine. Both apomorphine and L-DOPA induced a strong rotational behaviour which was registered in a specially designed rotometer" The direction of the rotation indicated that the denervated striatum was more sensitive to DA receptor stimulating drugs than the innervated striatum. This supersensitivity probably corresponded to the decentralisation type of supersensitivity in the peripheral nervous system although it developed faster The action of L-DOPA was inhibited by pretreatment with the DOPA-decarboxylase inhibitor Ro4-4602 which indicated that L-DOPA must be converted to DA in order to stimulate the supersensitive postsynaptic cells. Pretreatment with a single dose of reserpine also induced supersensitivity to apomorphine which reached its maximum on the 3rd day and then decreased on the 4th day Postsynaptic supersensitivity after degeneration of the nigro-striatal DA system is probably an important reason for the effectiveness of the L-DOPA therapy against Parkinson s disease.

Introduction

Supersensitivity after denervation is a well known phenomenon in the peripheral sympathetic nervous system. Although it is conceivable that a similar condition may develop after degeneration of central monoamine pathways, there are obvious difficulties to induce specific degenerations and to find

measurable variables to test. However the recent method of intracerebral, stereotaxic injection of 6-hydroxydopamine (6-OH DA) (Ungerstedt 1968 1971b) provides a technique of lesioning central noradrenaline (NA) and dopamine (DA) pathways with a high degree of specificity (Hökfelt and Ungerstedt 1971 Ungerstedt 1971b) Rats with large unilateral lesions of the nigro-striatal DA system are known to turn or even rotate after treatment with various drugs that interfere with the monoamine transmission (Andén *et al.* 1966a, 1967) This behaviour was reinvestigated after 6-OH DA lesions of the nigro-striatal DA system and a method of quantifying the behaviour in a specially designed "rotometer" was developed (Ungerstedt and Arbuthnott 1970) The rotational behaviour has been found highly reproducable and seems to reflect the degree of DA receptor stimulation (Arbuthnott and Ungerstedt 1970 Ungerstedt 1971d)

Andén *et al* (1966) noticed that some animals given L-DOPA rotated in a direction which indicated that L-DOPA was more active on the denervated side than on the innervated. They suggested that this might be due to denervation supersensitivity" Shibuya and Anderson (1968) found that the actions of 5-HTP in the chronic spinal cat were more rapid in onset and equally intense as in the acute spinal cat and attributed this to denervation supersensitivity after degeneration of the descending serotonin pathways. There are also signs of pharmacologically induced supersensitivity Dahlström *et al.* (1967) described supersensitivity after chronic treatment with reserpine and Dominic and Moore (1969) after treatment with α-methyltyronine.

The degeneration of the DA system has now been studied with the "6-OH DA rotation model" A number of parallels with the events in the peripheral nervous system were immediately apparent. The degeneration contraction of the nictitating membrane corresponded to a degeneration rotation and this was greatly potentiated by nialamide and amphetamine (Ungerstedt 1971d) In the present investigation the postsynaptic events during degeneration and pharmacological transmitter depletion are analysed by treatment with the DA receptor stimulating drug apomorphine (Ernst 1967 Andén *et al* 1967) or with L-DOPA

Material and Methods

About 150 male and 10 female Sprague Dawley rats have been used for the experiments. The body weights at the time of the stereotaxic operations were between 140—160 g During the course of the subsequent testing some animals acquired weights up to 450 g A few animals were tested over a period of two years.

Stereotaxic technique

All animals were operated in a David Kopf stereotaxic instrument. They were maintained under anaesthesia by a mixture of halothane, oxygen and nitrous oxide which was circulated through a mask tightly fitted over the nose of the animals. After a sagittal cut in the skin of the scull a 2 mm wide hole was drilled with an electrical trepan drill. Care was taken not to lesion the meninges. Electrodes or cannulas were vertically inserted. An antibiotic powder was applied locally before suturing. The animals usually woke up within 10 min after interruption of the anaesthesia.

Radiofrequency current was delivered by a Siemens Radiotom in order to produce electrocoagulations. The electrodes consisted of pointed stainless steel needles with a diameter of 0.25 mm. The insulation was removed 0.4—2 mm from the top depending upon the desired size of the lesion (see below). Unipolar electrocoagulations were performed with the indifferent lead inserted into the rectum. When performing bipolar lesions the current from the Radiotom was made symmetrical over an induction coil. The size of the different lesions were pretested *in vitro* in 37° C degree egg-white, stabilised with agar agar and the size was determined by a measuring microscope (Ungerstedt 1971a).

Intracerebral stereotaxic injections were made through a Hamilton cannula 0.2 mm in diameter. A PE20 polythene tubing connected the cannula to an "Agla micrometer all glass syringe" (Burroughs and Wellcome Co., England) which was driven by an electrical motor. The glass syringe was surrounded by ice. The fluid reached room temperature when passing through the tubing.

Electrocoagulations

Small unipolar electrocoagulations were placed unilaterally just rostral to the area ventralis tegmenti in order to lesion the ascending DA axons to the corpus striatum as described by Hökfelt and Ungerstedt (1969). Large bipolar electrocoagulations were performed unilaterally in the corpus striatum. The electrodes were positioned 1.5 mm apart one behind the other in a semi sagittal plane corresponding to the long axes of the nucleus. The insulation was removed at a distance of 2 mm from the tips. Except for a few controls all animals were lesioned on the left side.

6-hydroxydopamine injections

6-hydroxydopamine dihydrobromide (Regis) (6-OH DA) was dissolved in saline with ascorbic acid (0.2 mg/ml) added to prevent auto-oxidation. All doses below refer to the base. 6-OH DA was injected unilaterally into DA

neuron structures in the substantia nigra (8 μg/4 μl) the rostral area ventralis tegmenti (6 μg/3 μl) and the far lateral hypothalamus (6 μg/3 μl) and also caudal to the substantia nigra (8 μg/4 μl) Control injections were performed into the same areas with the exception that 6-OH DA was omitted from the solution. Injection speed was always 1 μl/min. The cannula was usually placed slightly to the side of the structure aimed for in order to avoid direct mechanical damage by the electrode tip. The outflow from the cannula was aimed in the desired direction. In case of injections into the substantia nigra and caudal to the substantia nigra the cannula was placed just rostral or caudal to the DA cell bodies and the outflow of the cannula was aimed caudally In case of the area ventralis tegmenti and lateral hypothalamus the cannula was positioned laterally or medially to the structure and the outflow was directed towards the DA axons.

Recording rotational behaviour

The unilaterally lesioned animals were tested in a rotometer (Ungerstedt and Arbuthnott 1970) for the ability of certain drugs to induce rotational behaviour A rotometer (fig 1) consists of a perspex half sphere within which the animal moves around. A light harness is fitted around the chest of the animal and a thin steel wire, connected to the harness, transfers the movements of the animal to a cam and a microswitch situated in the geometrical center of the sphere. The microswitch reacts to each full turn of the animal. The turns are registered on electromechanical counters. The counting stops automatically after two minutes and the third minute is used to write down manually the reading of the counter The automatic relay then zeroes the counter and the cycle is repeated. Six animals are recorded simultaneously and apart from the rotational speed the experimenter observes the behaviour of the animals. The rotational behaviour of the individual animal is expressed as the average rotational speed (turns/minute) for each three minute period. The average figure is then based on the two first minutes when the turns were counted. The mean rotational behaviour of 4—6 animals is calculated in order to evaluate the effect of a certain drug The curves are plotted as average turns per minute for a 6 or 12 minute period The standard error of the mean (S.E.M) is calculated for each plotted value. The rotational speed is expressed in positive y values if the animals rotate towards the operated side and in negative y values if the animals rotate towards the unoperated side. Dose response relationships are plotted as the total number of turns the animals make versus the dose of the drug Figure 1 explains this way of presenting the data.

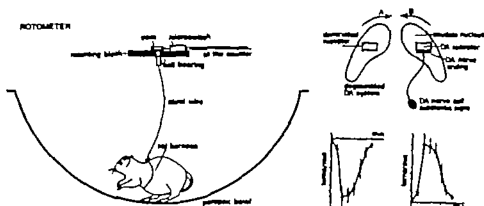


Fig. 1. *Left*. Schematic drawing of the rotometer. The movements of the rat are transferred by the steel wire to the microswitch arrangement.

Upper right. Principal outline of the experimental situation shown in a horizontal projection of the nigro-striatal DA system. When the stimulation of the denervated receptor dominates, the animal rotates in direction A. When stimulation of the innervated receptor dominates, the animal rotates in direction B.

Lower right: The rotational behaviour is presented as turns per min versus time. The curves are given negative y-values when stimulation of the denervated receptor dominates and positive y-values when stimulation of the innervated receptor dominates. Each point represents the mean ± S.E.M. of a certain number of animals.

Histochemistry

After the pharmacological testing (see below) representative animals from all groups (amounting to about 2/3 of all animals) were analysed with the Falck and Hillarp histochemical method for biogenic monoamines (Carlsson *et al* 1962. Falck 1962. Falck *et al*. 1962. Corrodi and Jonsson 1967). The animals were killed by decapitation during light chloroforme anaesthesia and the brains quickly dissected out, cut in four frontal sections and placed on stiff paper. A stiff nylon netting was placed on the top of the pieces to prevent them from falling apart during the subsequent freezing. Freezing was performed by dropping the pieces into liquid propane cooled by liquid nitrogen. The pieces were then transferred to a FT 1 freeze-drier (Bergman and Beving AB. Stockholm) freeze-dried for 2 1/2—3 1/2 days, reacted for 1 1/2 hour with gaseous formaldehyde in a preheated sealed desiccator which was placed in a 80° C oven. The pieces were then vacuum embedded for 10 min in liquid paraffine, sectioned, mounted in Entellane (Merck) with added xylol and studied in a fluorescence microscope. For details on the histochemical method and the freeze-drier see Fuxe *et al* (1970). Olson and Ungerstedt (1970a) and Unger

stedt (1971a) From a representative group of about 30 dissected animals thin slices were cut from both sides of the cortex. The slices were evenly smeared over object glasses with the help of a cover glass. The smears were dried over night in a desiccator treated with 80 C gaseous formaldehyde and studied in the fluorescence microscope. This method has been especially de signed to visualize cortical monoamine nerve terminals (Olson and Unger stedt 1970b)

Pharmacological experiments

A number of drugs were tested for their ability to induce rotational behaviour in the unilaterally lesioned animals. The animals were not tested earlier than one month after the operation except when specially indicated (see below) The various experiments are listed below under headings indicating the pur pose of the experiments. The following drugs were used apomorphine HCl (Sandoz, Basel) L-DOPA (Ajinomoto, Tokyo) reserpine (Swedish Ciba Stockholm) DL-α methyl tyrosine methylester HCl (H44/68 Hässle, Göteborg) haloperidol (Leo, Helsingborg) (H[1] (DL-seryl) N[2] (2,3 4-trihydroxy benzyl) hydrazine (Ro4-4602, Hoffman-La Roche, Basel) The doses given refer to the form indicated above except for apomorphine-HCl that was cal culated as the base. Haloperidol was dissolved in a few drops of glacial acetic acid, the final solution being made up with 5.5 % glucose solution. Apomorphine-HCl was dissolved in saline with added ascorbic acid (0 ? mg/ml) L-DOPA, H44/68 and Ro4-4602 were dissolved in saline All drugs were administered i.p

The anatomical structures are abbreviated in the following way SNR = substantia nigra AVT = area ventralis tegmenti LH = lateral hypothala mus CP = nucleus caudatus putamen = corpus striatum. For details of the site of a particular lesion see Ungerstedt (1971a) The number of animals is indicated within brackets.

Effects of various lesions

1 6-OH DA was injected into SNR (10) AVT (10) LH (6) and caudal to SNR (8) 2 Saline was injected into the same areas (12) 3 Electrocoagula tions were made in AVT (10) All animals received apomorphine, 0.25 mg/kg

Dose response

1 6-OH DA was injected into SNR (6) apomorphine 0.05 0.1 0.5 1 0 5 0 mg/kg was administered to all animals in a random order Testing was done every second day and each animal received each dose only once 2. 6-OH DA

was injected into SNR (5) L-DOPA 5 10 25 50 100 200 mg/kg was administered as apomorphine except that testing was done every third day

Time response

1 6-OH DA was injected into SNR (6) apomorphine 0.25 mg/kg was administered 12 hours, 1 2, 10 21 29 121 ?21 days after 6-OH DA. 2 6-OH DA was injected into SNR (6) L-DOPA was administered 1 2 9 23 104 days after 6-OH DA

Interaction with reserpine

1 Electrocoagulation in CP (6) reserpine 10 mg/kg + apomorphine 0.25 mg/kg 8 hours, 1 2 3 4 5 6, 8 21 47 119 days after reserpine. All animals were tested with apomorphine 0.25 mg/kg 2 days prior to the above experiments in order to obtain a control value

Denervation and pharmacological depletion

1 6-OH DA was injected into SNR (6) reserpine 10 mg/kg + apomorphine 0 25 mg/kg 4 and 48 hours after reserpine.
2 6-OH DA was injected into SNR (4) reserpine 2 mg/kg + H44/68 100 mg/kg ? hours after reserpine + apomorphine 0 25 mg/kg 2 hours after H44/68 All animals were tested with apomorphine 0.25 mg/kg 2 days prior to the above experiments in order to obtain a control value.

Effect of a neuroleptic

1 6-OH DA injected into SNR (5) apomorphine 0.25 mg/kg two days later haloperidol 1 mg/kg + apomorphine 0.25 mg/kg 30 min after haloperidol.

Effect of DOPA-decarboxylase inhibition

1 6-OH DA was injected into SNR L-DOPA 100 mg/kg (5) Ro4-4602 50 mg/kg + L-DOPA 100 mg/kg (4) Ro4-4602 250 mg/kg + L-DOPA 100 mg/kg (4) Ro4-4602 500 mg/kg + L-DOPA 100 mg/kg (4) Ro4-4602 1000 mg/kg + L-DOPA 100 mg/kg (4) L-DOPA 10 mg/kg (4) Ro4-4602 50 mg/kg + L-DOPA 10 mg/kg (2) Ro4-4602 500 mg/kg + L-DOPA 10 mg/kg (2) Ro4-4602 1000 mg/kg + L-DOPA 10 mg/kg (2) 2 6-OH DA was injected into SNR (3) apomorphine 0.25 mg/kg two days later Ro4-4602 500 mg/kg + apomorphine 0 25 mg/kg 3 Electrocoagulation in CP L-DOPA 100 mg/kg (4) Ro4-4602 50 mg/kg + L-DOPA 100 mg/kg (4) Ro4-4602 500 mg/kg + L-DOPA 100 mg/kg (4)

Correlation between the effect of apomorphine and L-DOPA

6-OH DA was injected into SNR (32) apomorphine 0 1 mg/kg From these animals a random group was selected and tested with L-DOPA 10 mg/kg (14) The total number of turns on apomorphine was compared to the total number of turns on L-DOPA and the correlation coefficient was calculated.

Results

Histochemistry

The unilateral electrocoagulations of the ascending DA axons were found to cause a complete degeneration of the DA nerve terminals in the corpus striatum. This corresponded to our earlier electron and fluorescence microscopical studies (Hökfelt and Ungerstedt 1969)

The unilateral electrocoagulation in the corpus striatum destroyed the whole nucleus except for its most caudal part. In most animals the lesion extended into the cortex and the globus pallidus.

The unilateral 6-OH DA injections into the substantia nigra degenerated virtually all DA cell bodies. In a few animals a couple of cells remained in the most lateral part of the nucleus. The DA nerve terminals in the corpus striatum disappeared, while there was a partial denervation of the olfactory tubercle and nucleus accumbens. In some animals the injection was too caudal and cells remained in the most rostral part of the nucleus. The corpus striatum then showed scattered nerve terminals. The results obtained from these animals were treated separately (see below) There was a strong retrograde accumulation of fluorescent material in the dorsal and ventral NA bundles (Ungerstedt 1971a, c) and there was a partial disappearance of the NA nerve terminals in the ipsilateral cortex and in the hypothalamus.

Unilateral 6-OH DA injections into the rostral area ventralis tegmenti and the lateral hypothalamus induced a strong retrograde accumulation of fluorescent material in the DA axons but also in the NA axons in the medial fore brain bundle The DA nerve terminals in the corpus striatum the olfactory tubercle and the nucleus accumbens disappeared almost completely The NA nerve terminals in the cortex disappeared ipsilaterally after 6-OH DA injection into the lateral hypothalamus, while there was only a small decrease after injections into the area ventralis tegmenti. There was a partial unilateral decrease in the NA nerve terminals in the preoptic area and the hypothalamus after both lesions.

The observed effects on the central monoamine neurons were thus consistent with what is described in other studies on the method of 6-OH DA induced degenerations (Ungerstedt 1971b) as well as on the anatomy of the

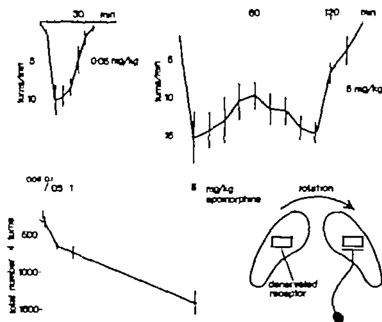


Fig 2. Apomorphine induced rotation in rats unilaterally denervated with 6-OH DA. Rotation curves are shown for the smallest and the largest dose. Each point represents the mean ± S.E.M. for 6 animals.

Lower left dose-response. *Lower right* horizontal outlin of th experimental situation in the igro-striatal DA system (see fig. 1)

monoamine pathways (Ungerstedt 1971a) The 6-OH DA lesions affected primarily NA and DA neurons while 5-hydroxytryptamine (5-HT) neurons were unaffected. The unspecific damage was restricted to a small area at the tip of the injection cannula.

Effects of arious lesions

Apomorphine (0.25 mg/kg) induced a strong rotation towards the unoperated side in all animals where the nigro-striatal DA system was degenerated i.e after 6-OH DA injection into the substantia nigra, the rostral area ventralis tegmenti and the lateral hypothalamus as well as after electrocoagulation in the rostral area ventralis tegmenti. No rotation occurred after 6-OH DA injection caudal to the substantia nigra when only NA axons were affected Apomorphine did not induce any rotation in animals where saline was in jected instead of the 6-OH DA solution.

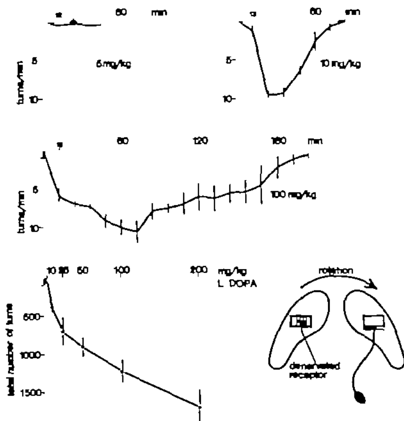


Fig 3 L-DOPA induced rotation in rats unilaterally denervated with 6-OH DA. Rotation curves are shown for three dose levels. Each point represents the mean ± S.E.M. for 5 animals.

Lower left dose response. *Lower right* horizontal outline of the experimental situation in the nigro-striatal DA system (see fig. 1)

A number of animals showed weak or no rotational behaviour after apomorphine The histochemical analysis revealed a misplacement of the injections and the results obtained from such animals were discarded.

Dose response (fig 2 3)

Apomorphine induced rotation towards the unoperated side and continuous sniffing in animals unilaterally lesioned by a 6-OH DA injection into the substantia nigra. After the lowest doses (0.05 and 0 1 mg/kg) there was a sharp increase in the speed of the rotation and a somewhat slower decline towards zero. After the larger doses, however the rotation reached a peak value shortly after the injection and another peak value shortly before the cessation of the

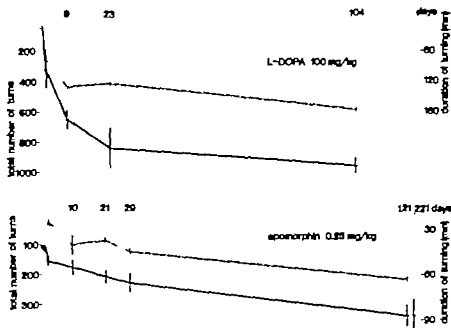


Fig. 4 Development of supersensitivity t L-DOPA and pomorphine. Each point represents the mean ± S.E.M. for 6 animals. The curves represent the duration of the rotational behaviour as well as the total number of turns.

rotational behaviour In between these peaks there was a period of slower rotation. This period of decreased rotational speed coincided with a period of vigorous twisting of the body towards the unoperated side. By this twisting the animals reached the side close to the hindlegs and the root of the tail where they performed an intensive scratching with their fore paws and grooming with their mouths. The intensity of this behaviour increased with the dose of apomorhpine. The licking or scratching with the teeth changed into a compulsive gnawing and biting After the highest dose (5 mg/kg) they actually hurt themselves by gnawing a wound in the side of their bodies. The twisting was not of a tonic nature, i.e. they were able to straighten their bodies. The behaviour obviously interferred with the speed of the rotation and the peak rotational speed was around 15 turns/min for all doses between 0.1—5 mg/kg This is also reflected in the dose response relationships where the total number of turns levels off with increasing doses of apomorphine.

L-DOPA induced rotation in the same way as apomorphine i.e. towards the unoperated side. The threshold dose was 10 mg/kg After 10 and 25 mg/kg there was a sharp rise and a slower decline in the rotational speed and the ani-

mals showed continuous sniffing as after apomorphine. After higher doses (50—200 mg/kg) the speed leveled off around 10 turns/min, while twisting and grooming increased However this behaviour never reached the same strength as after apomorphine.

Time response (fig 4)

Twelve hours after the injection of 6-OH DA into the substantia nigra apomorphine (0.25 mg/kg) did not induce turning while there was a sharp increase in the total number of turns 1 and 2 days after 6-OH DA This increase continued slowly over a period up to 121 days, while there was no significant increase between 121 and 221 days. The increase in total number of turns was mainly dependent upon an increase in the duration of the turning and not in its speed

The response to L-DOPA (100 mg/kg) was similar to the response to apomorphine. There was a sharp increase in the total number of turns over the first days and then a gradual leveling off Also in the case of L-DOPA there was an increase of the duration of the rotational behaviour over time

Interaction with reserpine (fig 5)

Before or eight hours after a single large dose of reserpine (10 mg/kg) the administration of apomorphine (0.25 mg/kg) did not induce any rotational behaviour in animals where the corpus striatum had been unilaterally lesioned. One two and three days after reserpine, however the animals showed a strong rotational behaviour towards their lesioned side. About 30 min after apomorphine the rotational behaviour declined and after a short transitional period the animals started to rotate in the opposite direction (fig 5) On the fourth day after reserpine there was a sudden decrease in the total number of turns on apomorphine in either direction. From the seventh day there was no longer any switch over in the direction of the rotation. The rotation then gradually decreased and on the 47th and 119th day it seemed to have reached the same values as before reserpine.

D nervation and pharmacological depletion (fig 6)

Four hours after reserpine (10 mg/kg) there was a decrease in the rotational behaviour after apomorphine (0.25 mg/kg) towards the non-lesioned side in animals unilaterally injected with 6-OH DA into the substantia nigra. However 48 hours after reserpine the rotational behaviour was back to control values. After reserpine (2 mg/kg) and H44/68 (100 mg/kg) there was also a decrease in the rotational behaviour as compared to control values but still a clear cut rotational behaviour

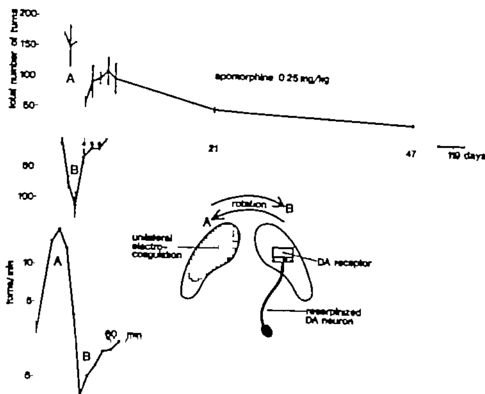


Fig 5 Supersensitivity after reserpine *Uppe curve* mean total number of turns in direction A and in direction B ± S.E.M. for 6 animals after treatment with pomorphine. Reserpine was administered at time zero.

Lowe left rotation curve from one representative animal on the 3 d day after reserpine. The animal shifted from direction A to direction B

Lower ight horizontal outline of the experimental situation in the lgro-striatal DA system.

Effect of neurol ptic (fig 6)

Pretreatment with haloperidol (1 mg/kg) strongly inhibited the apomorphine (0 25 mg/kg) induced rotational behaviour as compared to control values in animals injected with 6-OH DA into the substantia nigra.

Effect of DOPA-decarboxylase inhibition (fig 6—8)

Increasing doses of the DOPA-decarboxylase inhibitor Ro4-4602 (50—1 000 mg/kg) to animals injected with 6-OH DA into the substantia nigra caused an increasing delay of the onset as well as an increasing duration of the rota

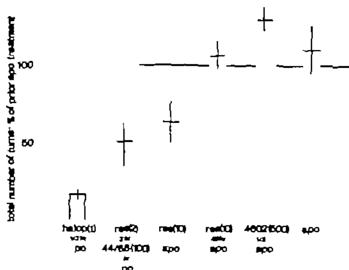


Fig. 6. Effects of various drugs on the apomorphine induced rotation in animals unilaterally denervated with 6-OH DA. The total number of turns are expressed as per cent of a treatment with apomorphine 2 days earlier. Each column represents the mean ±S.E.M. for 3—6 animals. The following drugs are abbreviated haloperidol, reserpine H44/68, Ro4-4602 and apomorphine. The dose of apomorphine was always 0.25 mg/kg, other doses are indicated in mg/kg within brackets. The time intervals between the administration of the different drugs are indicated.

tional behaviour after L-DOPA (100 mg/kg) (fig. 7). The same occurred when the rotational behaviour was induced with the border line dose of L-DOPA (10 mg/kg). The rotational behaviour after apomorphine (0.25 mg/kg) was slightly increased after pretreatment with Ro4-4602 (500 mg/kg) (fig. 6).

L-DOPA alone (100 mg/kg) did not induce any rotational behaviour in animals with unilateral electrocoagulation of the corpus striatum. However after pretreatment with Ro4-4602 (50 mg/kg) the animals rotated towards the lesioned side. If the dose of Ro4-4602 was increased to 500 mg/kg L-DOPA (100 mg/kg) did not induce rotation (fig. 8).

Correlation between the effect of apomorphine and L DOPA

When low doses of apomorphine (0.1 mg/kg) and L-DOPA (10 mg/kg) were compared on the same animals (6-OH DA injection into the substantia nigra) there was a close correlation between the total number of turns induced by the two drugs (Correlation coefficient = 0.76).

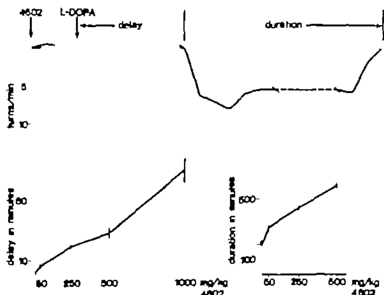


Fig. 7 Effect of the DOPA-decarboxylase inhibitor Ro4-4602 on L-DOPA induced rotation in animals unilaterally denervated with 6-OH DA. *Upper curve* principal diagram of the rotational behaviour Ro4-4602 is given at time zero. L-DOPA (100 mg/kg) is given 30 min later The delay until the onset of the rotational behaviour and its duration are measured.

Lower left: th delay after increasing doses of the inhibitor

Lower right the duration after increasing doses of the inhibitor Each point represents the mean ± S.E.M. for 4—5 animals.

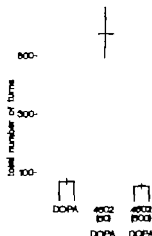


Fig. 8 Effect of the DOPA-decarboxylase inhibitor Ro4-4602 on L-DOPA (100 mg/kg) induced rotation in animals where the corpus striatum is unilaterally lesioned (see fig. 5) The dose of Ro4-4602 is indicated within brackets Each column represents the mean ± S.E.M for 4—5 animals.

Discussion

In a study on the functional significance of the nigro-striatal DA system (for references on the DA system, see Ungerstedt 1971a) Andén *et al.* (1966) showed that drugs interferring with the DA metabolism induced strong postural asymmetries in rats with lesions of the nigro-striatal pathway i.e. unilateral electrocoagulation of the axon bundle or removal of the corpus striatum. Release of DA by treatment with nialamide + reserpine or amphetamine (Andén *et al* 1967) induced vigorous rotation towards the lesioned side. This rotational behaviour thus seems to be due to an imbalance in the striatal DA activity of the two sides of the brain. This conclusion was further supported by the fact that direct unilateral injection of DA into the caudate nucleus produced contralateral turning or rotation if monoamine oxidase was inhibited (Ungerstedt *et al.* 1969)

The possibility to lesion the nigro-striatal DA system by direct intracerebral injections of 6-OH DA (Ungerstedt 1968 1971b) offered a way to remove the DA system unilaterally in a more selective and reproducible way than was possible with electrical or mechanical lesions. We were encouraged to try to quantify the rotational behaviour occurring after unilateral DA release and thus led to the construction of a "rotometer" (Ungerstedt and Arbuthnott 1970) The rat is allowed to move around within a perspex half sphere while connected by a thin wire to the geometrical center of the sphere (fig 1) The movements of the animal are transferred by the wire to a microswitch arrangment in the center point. It is thus possible to record the number of turns per unit of time that is performed by a rotating rat. The behaviour is reproducable and dose dependent when the DA release is induced by amphetamine (Ungerstedt and Arbuthnott 1970) Andén *et al.* (1966) reported that DOPA sometimes induced rotation in the opposite direction to what would be expected by a release of DA from the intact side and suggested that the phenomenon could be due to a denervation supersensitivity after a lesion of the nigro-striatal DA system. In this paper the 6-OH DA rotation model" is used to test this effect of DOPA quantitatively and especially the effect of apomorphine which is known to stimulate DA receptors (Andén *et al.* 1967 Ernst 1967) The nigro-striatal DA system is unilaterally removed with comparatively little damage to other neuron systems (Hökfelt and Ungerstedt 1971 Ungerstedt 1971b) when 6-OH DA is administered into the DA cell group in the substantia nigra or into the ascending bundle of DA axons. This way of unilateral application of 6-OH DA into the substantia nigra denervates the corpus striatum without interference with the postsynaptic cell and its DA receptor A dominance in the DA receptor activity in the striatum on one

side forces the animal to rotate towards the other as is shown after direct unilateral striatal application of DA or apomorphine (Ungerstedt *et al* 1969) In the case of the unilaterally 6-OH DA denervated animals the rotational behaviour thus reflects a comparison between the effect of a certain drug on the innervated and the denervated side. The speed, duration and direction of the rotational behaviour results from a substraction of the effects on the two sides The only way to study the effect of DA receptor stimulation on one side only is to damage the whole striatal complex on the other This was done by unilateral electrocoagulation of the corpus striatum

The effect of apomorphine (0.25 mg/kg) was first tested on animals where lesions of the nigro-striatal DA system were produced at various levels along the DA pathway The necessary parameters for these lesions were provided by recent mapping studies on the rat brain monoamine pathways (Ungerstedt 1971a) as well as by standardisation of the 6-OH DA lesion method. The electrocoagulation of the DA axons has been investigated earlier by electron- and fluorescence-microscopic studies (Hökfelt and Ungerstedt 1969) Apomorphine induced rotation towards the non lesioned side in all animals except when 6-OH DA was injected into the ascending NA axons caudal to the substantia nigra. These animals did not show any rotational behaviour after apomorphine. In all rotating animals the common feature was a degeneration of the nigro-striatal DA system. The fact that rotational behaviour could be induced after lesions at various levels of the nigro-striatal DA system indicates that the response actually is elicited as a result of a degeneration of this system. Furthermore the direction of the rotation shows that the denervated side is most sensitive to apomorphine. The fact that haloperidol which blocks DA and NA receptors (Carlsson and Lindqvist 1963 Andén *et al* 1970) is able to inhibit the rotational behaviour is further evidence for the specificity of the response (fig 6)

In the dose-response experiment it became apparent that the denervated side reacted to very low doses of apomorphine. A clearcut rotation occurred alerady after 0 05 mg/kg of the drug (fig 2) The dose where apomorphine induced any comparable motor activity in normal animals wat at least 10—20 times higher Apomorphine is known to induce stereotyped behaviour (*e g* sniffing, gnawing) in normal animals (Amsler 1923 Ernst 1967 Randrup and Munkvad 1968) In the rotating animals stereotyped sniffing occurred simultaneously with the rotational behaviour and after higher doses it changed into a behaviour similar to the compulsive gnawing seen after high doses of apomorphine in normal animals. The onset of this behaviour is reflected in the rotational curves as a leveling off or even a decrease in the rotational speed at a time when the plasma concentration of the drug probably reaches its

highest value. Apart from the obvious disturbances of the rotational behaviour caused by the stereotyped oral behaviour there may be a counterbalance of the rotation at this point because of a competetive effect of apomorphine on the innervated side especially as the effect on the denervated side has reached a point where stereotyped oral behaviour is favorured over rotational behaviour *i.e.* an increase in the DA receptor stimulation results in a shift from locomotion and sniffing towards licking and gnawing (Fuxe and Ungerstedt 1970)

L-DOPA induced a rotation in the same direction as apomorphine *i.e.* towards the non-lesioned side which shows that L-DOPA, like apomorphine, is most effective on the denervated side (fig 3) Stereotyped sniffing occurred in the same way as after apomorphine but the compulsive oral behaviour seemed less pronounced after L-DOPA. However it was difficult to make a correct comparison as the threshold dose for L-DOPA (10 mg/kg) was higher than the highest dose tested for apomorphine. The leveling off effect after higher doses was obvious also after L-DOPA. While rotational behaviour occurred after 10 mg/kg of L-DOPA most other authors report that doses between 200—1000 mg/kg are necessary to induce any increased motor behaviour in normal animals (*e.g.* Randrup and Munkvad 1966 Ernst 1967) However Butcher and Engel (1969a, b) have shown that the peripheral actions of physiologically active DOPA catabolites may interfere with the central effect of the drug It is, thus, impossible to make a direct comparison between the effect of L-DOPA in the normal and in th DA denervated animal However the potentiation of the DOPA effect after denervation may well be in the same range as the potentiation of the apomorphine effect.

The development of the increased sensitivity to apomorphine and DOPA coincides with the disappearance of DA from the corpus striatum as found in fluorescent microscopic (Ungerstedt 1971b) as well as in electron microscopic studies (Hökfelt and Ungerstedt 1971) The great majority of DA nerve terminals loose their DA content 24—48 hours after the injection of 6-OH DA into the substantia nigra. The time responce curves for apomorphine and L-DOPA show the greatest increase in sensitivity during this period (fig 4) This increase continues over at least a month and in the case of apomorphine there might be an even further increase. In the case of both apomorphine and L-DOPA there is a definite increase in the duration of the rotation over time. It is mainly an increased duration of the rotational behaviour which is responsible for the increased total number of turns at the longer time intervals studied.

Degeneration of the DA nerve terminals involves several changes in the neuronal environment in the corpus striatum. We have attempted to separate and evaluate one variable *i.e.* the decreased amount of DA reaching the

postsynaptic cell This state can be induced by treatment with reserpine which depletes the nerve terminals of their content of DA. The corpus striatum was unilaterally removed by electrocoagulation and the animals were tested on apomorphine (0.25 mg/kg) in the rotometer (fig 5) The animals showed no rotational behaviour which indicated that the normally innervated post synaptic cells in the remaining corpus striatum did not respond to this low dose of apomorphine The animals wer then regularly tested on apomorphine after a single dose of reserpine (10 mg/kg) The animals showed no rotational behaviour 8 hours after reserpine However after 1 day apomorphine induced clearcut rotation towards the lesioned side which indicated an increased sensitivity to apomorphine. The sensitivity increased over the first three days after reserpine but showed a sudden decrease on the fourth day The response then slowly decreased Twenty-one days after reserpine the animals still rotated but when tested on the 47th and 119th day the response seemed to have reached the pre-reserpine level The rotational behaviour was complicated by the fact that, beginning on the 2nd day the animals shifted the direction of the rotation about 30 minutes after apomorphine The number of turns in the first direction, however was much higher than in the second direction This surprising behaviour was very reproduceable in all animals. A tentative explanation will be discussed in relation to the next series of experiments (see below)

From these experiments it seem probable that lack of DA reaching the postsynaptic cells is in itself able to induce an increase in the sensitivity of apomorphine. The condition is reversible as the sensitivity decreases when the DA neurons recover from reserpine. The strong sedation after reserpine is relieved already on the second and third day It is interesting to note that the increased sensitivity to apomorphine reaches its maximum at this time. It may be speculated that the early behavioural recovery is due to a strongly increased sensitivity to the small amount of DA that reaches the postsynaptic cell. The change in sensitivity on the fourth day probably reflects a sudden increase in the release of DA possibly because new or recowered storage granules are made available.

In order to compare an acute DA depletion with a chronic denervation reserpine (10 mg/kg) was given to animals with unilateral 6-OH DA induced degenerations of the nigro-striatal DA system. The rotational behaviour after apomorphine (0.25 mg/kg) was decreased 4 hours after reserpine but close to pre reserpine levels after 48 hours (fig 6) The low rotation in the 4 hour animals are probably due to the serious sedation as it is known from the previous experiment that no increased sensitivity to apomorphine is detected even 8 hours after reserpine. On the other hand, 48 hours after reserpine there

is an increased apomorphine sensitivity as demonstrated above and the animals have recovered from their strong sedation. Any decreased rotation on apomorphine at this time should then reflect the competition between the denervated and the depleted side as they tend to force the animal in opposite directions. However the response to apomorphine was back to normal again 48 hours after reserpine. There is no immediate explanation to this. Even after simultaneous depletion by reserpine (2 mg/kg) and synthesis inhibition with H44/68 (100 mg/kg) the denervated side dominates and forces the animals to rotate towards the non lesioned side. These experiments indicate that the acute lack of DA is not the only factor in determining the postsynaptic sensitivity to apomorphine. Acute depletion of DA does not induce the same level of increased sensitivity as denervation The duration of the period of no DA release and the actual removal of the DA nerve terminals are factors whose relative importance need to be settled.

It is also important to realise that the response to apomorphine after reserpine treatment may not only reflect changes in postsynaptic sensitivity to apomorphine but also in the interaction between NA, DA and 5-HT neurons. It is, in fact, known that a decrease in the NA transmission enhances the rotational behaviour (Ungerstedt 1971d) Some of the unexplained results above may be due to such phenomena.

The increased response to L-DOPA after denervation seems paradoxical as degeneration of the DA nerve terminals causes a decrease in the neuronal DOPA-decarboxylase and thus an impaired ability to synthesize DA from the exogenous L-DOPA (Andén *et al* 1966 Goldstein *et al.* 1969) In order to exclude an effect of L-DOPA itself on the postsynaptic cell, animals with 6-OH DA lesions of the nigro-striatal DA system were treated with the DOPA decarboxylase inhibitor Ro4-4602 before L-DOPA (Bartholini *et al* 1969) The delay in the onset of the rotation as well as its duration was progressively increased with increasing doses of Ro4-4602 (fig 7) The fact that a corresponding effect was not seen after Ro4-4602 and apomorphine indicate that an inhibition of DA synthesis is actually responsible for the effect on the L-DOPA induced rotation (fig 6) The delay in the onset of the rotation propably reflects the period of complete synthesis inhibition in the denervated caudate nucleus. The increased duration may similarly reflect a partial synthesis inhibition during which DA is slowly synthesized from a large pool of non-utilized L-DOPA. It is interesting to note that there is a delay in the onset of the rotation even after 50 mg/kg of Ro4-4602 This dose is generally thought to inhibit the peripheral decarboxylase but regarded as ineffective in the central nervous system (Bartholini *et al* 1969) However 50 mg/kg of Ro4-4602 inhibits the DOPA decarboxylase in the pericytes of the capillary

walls (Constantinidis *et al* 1968) and the results may thus, indicate that the pericytes constitute one functionally important site of decarboxylation in the denervated striatum The innervated corpus striatum reacted very differently as compared to the denervated L-DOPA (100 mg/kg) did not induce any rotation in animals with unilateral electrocoagulation of the corpus striatum, *i.e.* it did not affect the remaining innervated nucleus (fig 8) After inhibition of the peripheral decarboxylase with Ro4-4602 (50 mg/kg) the animals showed rotational behaviour but this was inhibited if the Ro4-4602 dose was increased to 500 mg/kg

These experiments demonstrate that L-DOPA itself is not able to stimulate the denervated postsynaptic cells directly and the increased response to L-DOPA is in fact an increased sensitivity to DA, synthesized from the exogenous L-DOPA. The site of this conversion is not known but the caudate nucleus still contains DOPA-decarboxylase after a degeneration of the nigro-striatal DA system (Andén *et al.* 1966 Goldstein *et al* 1969) The pericytes in the walls of the capillaries are known to contain DOPA-decarboxylase (Bertler *et al.* 1963 Constantinidis *et al* 1968) and it may possibly exist in the special catecholamine accumulating cell type that has been observed in the denervated caudate nucleus (Hökfelt and Ungerstedt 1969) 5-HT nerve terminals may constitute another probable site of decarboxylation

The phenomenon of supersensitivity after degeneration is well understood in the peripheral sympathetic nervous system and it is of obvious interest to relate this to the present findings. In the case of the nictitating membrane a few well defined events occur after extirpation of the superior cervical ganglion, *i.e.* loss of the endogenous transmitter and development of degeneration contraction, denervation supersensitivity and decentralisation supersensitivity The loss of DA during degeneration of the nigro-striatal DA system and the functional phenomena connected with this are discussed elewhere (Ungerstedt 1971) It may be sufficient to mention just a few observations. The DA nerve terminals in the striatum loose their transmitter content between 24—28 hours after axotomy (Hökfelt and Ungerstedt 1969 Ungerstedt 1971) Thus results at the most in a weak rotation towards the non-lesioned side. However the rotation may be potentiated greatly with a monoamine oxidase inhibitor (nialamide) Thus rotation is probably analogous to the degeneration contraction of the nictitating membrane (Langer 1966) The denervation supersensitivity starts to develop just before the onset of the degeneration contraction in the nictitating membrane and is fully developed shortly after the maximum of the degeneration contraction has passed (Langer and Trendelenburg 1966) The decentralisation supersensitivity on the other hand, develops gradually for at least four weeks (Langer *et al.* 1967) When

the supersensitivity of the DA system to apomorphine or L-DOPA is considered, it is apparent that it develops too late to be analogous to denervation supersensitivity to L-noradrenaline while it is strinkingly similar to the decentralisation type of supersensitivity although it appears somewhat too early to be strictly identical. It seems reasonable to assume that the sensitivity to apomorphine should be compared to the methoxamine sensitivity of the denervated nictitating membrane (Trendelenburg et al. 1970) Neither methoxamine nor apomorphine are thought to be inactivated by the membrane uptake mechanism and should therefore reflect the change in sensitivity of the postsynaptic cell It is obvious that this component of the supersensitivity develops faster after degeneration of the DA system as compared to the peripheral NA system. This is particularly evident in the case of a single dose of reserpine which does not induce supersensitivity in the nictitating membrane in contrast to what happens in the striatum (Fleming and Trendelenburg 1961)

The fact that postsynaptic supersensitivity develops after degeneration of the nigro-striatal DA system and after injection of reserpine has several implications. The increased response to exogenous L-DOPA is, of course, particularly interesting in connection with Parkinson's disease. It seems probable that the postsynaptic supersensitivity constitutes the physiological basis for the effectiveness of the L-DOPA therapy Furthermore, the 6-OH DA rotation model may prove valuable for the testing and detection of therapeutic agents effective against Parkinson's disease.

The supersensitivity after reserpine, and its time course, is interesting in view of the postulated functional pool of transmitter The argument is partly based on the recovery after reserpine. It may be that the existence of postsynaptic supersensitivity has made us overestimate the ability of the existing pool of transmitter to keep up normal function in the DA system.

Acknowledgement

This work was supported by grant B71 14X 715-06C from the Swedish Medical Research Council and by grants from Svenska Livförsäkringsbolags Nämnd för Medicinsk Forskning and Ollie and Elof Ericssons Stiftelse. I am greatly indebted to Miss Eva Henriksson and Miss Kerstin Sterner for their excellent technical assistance

References

Amsler C., Beiträge zur Pharmakologie des Gehirns. *A ch p Path Pharm* 1923 97 1—14

Andén N E., S. G Butcher H. Corrodi, K. Fuxe and L Ungerstedt, Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *E ope J Pharmacol.* 1970. *11* 303—314

Andén, N E., A. Dahlström, K. Fuxe and K. Larsson, Functional role of the nigro-neostriatal dopamine neurons *A ta ph rmacol (Kbh)* 1966a. *24* 263—274

Andén, N.-E., A. Dahlström, K. Fuxe K. Larsson I Olson and U Ungerstedt, Ascending monoamine neurons t the telencephalon and diencephalon. *Act physiol. scand* 1966b. *67* 313—326.

Andén, N E., Rubenson A., K. Fuxe and T Hökfelt, Evidence for dopamine recept stimulation by apomorphine *J Pharm Pharmacol.* 1967 *19* 6 7—629

Bartholini, G and A. Pletscher Cerebral accumulation and metabolism of C[1] DOPA after selective inhibition of peripheral decarboxylase. *J Pharmacol p Ther* 1968. *161* 14—20.

Bertler A B. Falck and E. Rosengren, The direct demonstration of a barrier mechanism in the brain capillaries. *A t pharmacol (Kbh)* 1963 *20* 317—321

Butcher L. L. and J Engel, Behavioral and biochemical effects of L-DOPA after peripheral decarboxylase inhibition. *Brai Res.* 1969a. *15* 233— 42.

Butcher L. L. and J Engel, Peripheral factors in the mediation of the effects of L-dopa on locomotor activity *J Pharm Ph rmac* 1969b. *21* 614—616.

Carlsson, A., B. Falck and N A. Hillarp, Cellular localization of brain monoamines. *A t phy iol sc nd* 1962. *56* Suppl. 196. 1—28.

Carlsson, A. and M. Lindqvist, Effect of chlorpromazine or haloperidol on formation of 3-methoxytyramine and normetanephrine in mouse brain. *Act pharmacol. (Kbh)* 1963 *20* 140—144

Constantinidis, J G Bartholini, R. Tissot and A. Pletscher Accumulation of dopamine in the parenchyma after decarboxylase inhibition in the capillaries of brain. *Ex perienti* 1968. *24* 130—131

Corrodi, H and G. Jonsson, The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines A review on the methodology *J Histochem Cytochem* 1967 *15* 65—78

Dominic, J A. and K. E. Moore Supersensitivity to the central actions of drenergic drugs following discontinuation of a chronic diet of a-methyltyrosine. *Psychopharmacologia (Berl)* 1969 *15* 96—101

Dahlström, A., K. Fuxe, B Hamberger and T Hökfelt, Uptake and storage of catecholamines in rabbit brain after chroni reserpine treatment. *J Ph rm. Pharmac* 1967 *19* 345—349

Ernst, A. M., Mode of action of apomorphine and dexamphetamine on gnawing compulsion in rats. *Psy hopharmacologia (Berl.)* 1967 *10* 316—323

Falck, B Observations of the possibilities of the cellular localization of monoamines by fluorescence method. *Act physiol. and* 1962 *56* Suppl. 197 1—26.

Falck, B N A. Hillarp, G Thieme and A. Torp, Fluorescence of catecholamines and related compounds condensed with formaldehyde. *J Histochem Cytochem* 1962. *10* 348—354

Fleming, W W and U Trend lenburg, Development of supersensitivity to norepine

phrine after pretreatment with reserpine *J Pharmacol. exp Ther* 1961 *133* 41—51
Fuxe K. T Hökfelt, G Jonsson, and U Ungerstedt, Fluorescence microscopy in neuroanatomy *In* "Contemporary research methods in neuroanatomy" Eds. W Nauta and S. Ebbesson Springer 1970 275—314
Fuxe, K. and U Ungerstedt, Histochemical biochemical and functional studies on central monoamine neurons after acute and chronic amphetamine administration. *In* Amphetamines and related compounds Eds E. Costa and S Garattini. Raven Press, New York. 1970. 257—288
Goldstein, M., B. Anagnoste A. F Battista, W S Owen and S Nakatani Studies of amines in the striatum in monkeys with nigral lesions. The disposition, biosynthesis and metabolites of [3H] dopamine and [14C] serotonin in the striatum. *J Neurochem* 1969 *16* 645—653
Hökfelt, T and U Ungerstedt, Electron and fluorescence microscopical studies on the nucleus caudatus putamen of the rat after unilateral lesions of ascending nigroneostriatal dopamine neurons. *Acta physiol. scand.* 1969 *76* 415—426
Hökfelt, T and U Ungerstedt, Effects of 6-hydroxydopamine on central monoamine neurons with special reference to the nigro-striatal dopamine system: An electron and fluorescence microscopical study *Brain Res* 1971 In press.
Langer S. Z., The degeneration contraction of the nictitating membrane in the unanesthetized cat. *J Pharmacol exp Ther* 1966 *151* 66—72
Langer S. Z., P R. Draskoczy and U Trendelenburg, Time course of the development of persensitivity to various amines in the nictitating membrane of the pithed cat after denervation or decentralisation. *J Pharmacol. exp Ther* 1967 *157* 255—273
Langer S. Z. and U Trendelenburg, The onset of denervation supersensitivity *J Pharmacol. exp Ther* 1966 *151* 73—86.
Olson, L. and U Ungerstedt, A simple high capacity freeze-drier for histochemical use *Histochemie* 1970a. *22* 8—19
Olson, L. and U Ungerstedt, Monoamine fluorescence in CNS smears sensitive and rapid visualization of nerve terminals without freeze-drying *Brain Res* 1970b. *17* 343—347
Randrup A. and I. Munkvad, Role of catecholamines in the amphetamine excitatory response *Nature* 1966. *11* 540
Randrup A and I Munkvad, Behavioural stereotypies induced by pharmacological agents *Pharmakopsychiat Neuro-Psychopharmacol.* (Stuttgart) 1968. *1* 18—26
Shibuya, T and E. G Anderson, The influence of chronic cord transection on the effects of 5-hydroxytryptophan l-tryptophan and pargyline on spinal neuronal activity *J Pharmacol exp Ther* 1968. *164* 185—190.
Trendelenburg U R. A. Maxwell and S. Pluchino, Methoxamine as a tool to assess the importance of intraneuronal uptake of l-norepinephrine in the cat nictitating membrane *J Pharmacol exp Ther* 1970. *172* 91—99
Ungerstedt U 6-Hydroxydopamine induced degeneration of central monoamine neurons. *Eur J Pharmacol* 1968 *5* 107—110.
Ungerstedt U Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta physiol scand* 1971 Suppl. 367 1—48
Ungerstedt U Histochemical studies on the effects of intracerebral and intraventricular injections of 6-hydroxydopamine on monoamine neurons in the rat brain. *In* "6-Hydroxydopamine and catecholamine neurons" Eds. T Malmfors and H Thoenen.

North-Holland. 1971b. 101—127
Ungerstedt U Noradrenaline pathway in the rat brain Principal architecture *Scienc* 1971c. In press.
Ungerstedt, U Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behaviour *Act physiol. sc d* 1971d. Suppl. 367 49—68
Ungerstedt U and G Arbuthnott, Quantitative recording of rotational behaviour in rats after 6-hydroxy-dopamine lesions of the nigro-striatal dopamine system. *Brai Res.* 1970. *24* 485—493
Ungerstedt, U L. L. Butcher S. G Butcher N E Andén and K. Fuxe, Direct chemical stimulation of dopaminergic mechanism in the neostriatum of the rat. *Brain Res* 1969 *14* 461—471

From the Department of Histology, Karolinska Institutet, Stockholm, Sweden

Adipsia and Aphagia after 6-Hydroxydopamine Induced Degeneration of the Nigro-striatal Dopamine System

By

U. Ungerstedt

Abstract

The functional role of the nigro-striatal dopamine (DA) system has been investigated on the basis of a recent detailed mapping of its path and a new method of lesioning the catecholamine systems selectively by intracerebral injection of 6-hydroxydopamine (6-OH-DA). The investigation was especially focused on the symptoms of adipsia, aphagia, hypokinesia and catalepsia after lateral hypothalamic lesions as such lesions may interrupt the ascending DA axons. Electrocoagulations or 6-OH-DA lesions were performed bilaterally at several sites along the DA pathway and the behavioural effects were evaluated in relation to the histochemically detected lesion of the DA pathway. It was concluded that bilateral complete degeneration of the nigro-striatal DA pathway produces severe, long lasting adipsia and aphagia, hypoactivity, difficulties to initiate activity and loss of exploratory behaviour and curiosity. Experiments with DA receptor stimulating and blocking drugs supported the lesion results. Catalepsia and somnolence were attributed to the interruption of other pathways. The results suggest an important role for the nigro-striatal DA system and the striatum in the control of behaviour. A number of symptoms earlier related to the hypothalamus may in fact be due to disturbance of the nigro-striatal DA system.

Introduction

The existence of an uncrossed monosynaptic, dopaminergic* pathway arising in the substantia nigra and terminating in the neostriatum is well established from histological (Andén *et al.* 1964, Andén *et al.* 1965, Andén *et al.* 1966, Llamas and Rainoso-Suarez 1969), biochemical (Andén *et al.* 1964, Poirier and Sourkes 1965, Goldstein *et al.* 1966, Faull and Laverty 1969) and

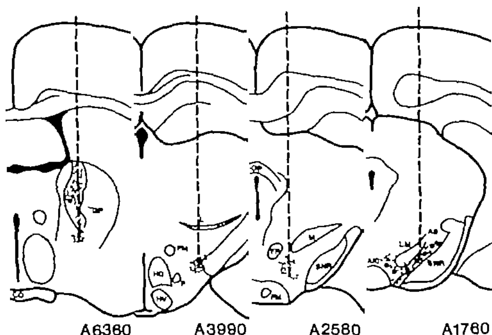


Fig. 1 The nigro-striatal DA pathway shown in representative frontal sections of the rat brain. The number refer to the frontal planes of the König and Klippel atlas (1963)

A1760 The DA cell bodies (large dots) in th substantia nigra (group A9) send their axons medially to join the axons from the DA cell bodies situated dorsal to th inter peduncular nucleus (group A10) The dotted line indicates the sit f the 6-OH DA injection cannula.

A2580 DA axons assembled in dense bundle rostral to the area ventralis tegmenti. The dotted line indicates the site of the 6-OH DA injection cannula or the coagulation electrode

A3990 DA axons assembled in the lateral hypothalamus. The medial part of the bundle belongs to th meso-limbic DA system. The lateral part belongs to the nigro-triatal DA system. The dotted line indicates the site of the 6-OH DA injection cannula or coagulation electrode in the lateral hypothalamus.

A6360 The meso-limbic DA axons are located t the tip of the internal capsule The nigro-striatal DA axons ascend through the internal capsule, spread out in th globus pallidus and enter the corpus striatum.

Abbreviations CAI Capsula interna, CO chiasma opticum, F fornix, FMT: Fasciculus mamillothalamicus, FR Fasciculus retroflexus, GP globus pallidus HA, nucleus anterior (hypothalami) HD nucleus dorsomedialis (hypothalami) HV: nucleus ventromedialis (hypothalami) IP Nucleus interpeduncularis, LM, lemniscus medialis SNR substantia nigra, zona reticulata.

Table 1

Experimental group	Lesions (bilateral)	No. of animals	No. of adipsic and aphagic animals	
1	Lateral hypothalamus electrocoagulation	17	14	Acute animals not supported by tube feeding. Killed after 3—5 days, if adipsia or phagia developed.
2	Lateral hypothalamus 6-OH DA injection	4	4	
3	Substantia nigra 6-OH DA injection	18	10	
4	Caudal to substantia nigra 6-OH DA injection	10	0	
5	Rostral hypothalamus electrocoagulation	1	0	
6	Rostral hypothalamus 6-OH DA injection	8	0	
7	Lateral hypothalamus electrocoagulation	7	7	Chronic animals supported by tube feeding. Killed after 3—8 weeks.
8	Lateral hypothalamus 6-OH DA injection	6	4	
9	Area ventralis tegmenti electrocoagulation	4	4	
10	Area ventralis tegmenti 6-OH DA injection	4	4	
11	Substantia nigra 6-OH DA injection	1	6	
12	Same sites as above saline injected control animals	8	0	

In order to determine water and food consumption the animals were housed in individual cages. Water was provided through inverted bottles with drinking nipples. The bottles were weighed in order to find out the amount of water consumed. It was determined that occasional water droplets being lost from the bottles during handling never constituted more than 0.6 ml. Ground food was placed in jars on the bottom of the cages. Food consumption was determined by weighing the jars with their remaining food contents as well as the occasional food spillings on the trays beneath the wire floors. Body weight was determined daily. In the lesion experiments the

animals were given food and water *ad libitum*. Animals in group 7—11 were tube fed if necessary from the second day by intragastric injection of water Aminolipide (Vitrum) and Mentene. They were nursed with great care in order to maintain their weights.

The behaviour of all the operated animals was carefully observed. Special attention was devoted to spontaneous activity and motor ability. The animals were regularly tested for catalepsia in the way described by Balagura *et al* (1969) i.e. the paws were placed one by one on a small box and the time during which the rat kept its paw on the box was registered with a stop watch. Apart from observations of the spontaneous activity of the animals their activity was registered in an Animex activity box (Farad electronics, see Svensson and Thieme 1969) for 15 minute periods every day during the first week after the operation. Some animals of particular interest were registered continously for 24—48 hours. Normal unoperated or saline injected animals were used as controls in the experiments (group 1?)

Five animals in group 3 (6-OH DA injection into the substantia nigra) were tested on apomorphine (5 mg/kg) on the third day after the operation shortly before being sacrificed.

In a final series of experiments rats were kept on a 23½ hours water deprivation schedule. Food was then always available. The animals were treated with Pimozide which is known to block DA receptors in the central nervous system (Andén *et al.* 1970) and apomorphine which stimulates DA receptors (Andén *et al.* 1967 Ernst 1967) Pimozide in doses of 1 mg/kg, 2 mg/kg, 5 mg/kg and 10 mg/kg was given 4 hours before the drinking period and apomorphine in doses of 0.1 mg/kg, 0.2 mg/kg, 0.5 mg/kg and 1 mg/kg 3 minutes before. These time periods were selected in order to achieve an optimal drug action at the time of water consumption. Pimozide was dissolved in 1—3 droplets of HAc and then diluted in isotonic saline. Control animals received saline with added HAc. Apomorphine was dissolved in saline. All injections were made i.p.

The brains from most of the lesioned animals (see below) were analysed with the *histochemical fluorescence method* for monoamines. The animals were decapitated during light chloroforme anaesthesia. The brains were rapidly dissected out, cut in four frontal sections, placed on labeled pieces of stiff paper frozen in liquid propane which was cooled to −190 C by liquid nitrogen. The frozen brain pieces were transferred to a histochemical freeze-drier (FT 1 Bergman and Beving, Stockholm) and processed for ? 1/2—3 1/2 days. The dried pieces were reacted for 1 1/2 hours with gaseous formaldehyd in a closed vessel which was placed in a 80° C oven. The formaldehyde gas was generated from paraformaldehyde powder placed on the bottom of the vessel. The

brain pieces were then vacuum imbedded in paraffine sectioned and mounted. The brain sections were finally examined in a Zeiss fluorescence microscope (Falk *et al* 1962 for further references on the histochemical method and the freeze drier see Fuxe *et al* 1970 Olson and Ungerstedt 1970)

Results

Histochemistry of the lesions

The Falck and Hillarp fluorescence histochemical technique permits the visualisation of cellularly localised noradrenaline (NA) dopamine (DA) and 5-hydroxytryptamine (5-HT) The NA and DA containing nerve cell bodies and nerve terminals develop a strong green, to yellow-green fluorescence, while 5-HT containing nerve cell bodies and nerve terminals show a less strong yellow fluorescence. The NA, DA and 5-HT axons contain too little monoamines to be clearly visualised. For a full account of the histochemical technique, see Fuxe *et al* 1970. When the monoamine cell bodies are lesioned there is an anterograde degeneration of the whole neuron and consequently a disappearance of the nerve terminals. In case of the DA neurons the nerve terminal fluorescence disappears within 2 days (Hökfelt and Ungerstedt 1969) while the NA and 5-TH nerve terminal fluorescence disappears within 6—10 days (Ungerstedt unpublished) If the monoamine neurons are lesioned somewhere along their axons or even within the terminal areas there is an interruption of the axonal flow of the transmitter and thus a pile up of fluorescent material in the axons (Dahlström and Fuxe 1964) which may then be detected (fig. 2) Various lesions of the monoamine neurons have been very helpful when mapping these neurons in the rat brain (Ungerstedt 1971a)

In this investigation the monoamine pathways have been interrupted by electrocoagulations and 6-OH DA injections. In the final examination of the rat brains it was thus possible to detect the detailed effects of these lesions by the pile up of fluorescence in severed axons and the disappearance of fluorescence in nerve terminals and cell bodies.

The electrocoagulation destroyed everything within the coagulated area. The 6-OH DA injections, on the other hand, induced only a small unspecific damage at the tip of the injection cannula. The sorrounding brain parenchyma showed normal appearance, except for DA cell bodies and NA and DA axons and nerve terminals that degenerated after the intracerebral injection of 6-OH DA. The effects of intracerebral injections of 6-OH DA have been investigated in a series of papers (Ungerstedt 1968 1971b Hökfelt and Ungerstedt 1971)

animals were given food and water *ad libitum*. Animals in group 7—11 were tube fed if necessary from the second day by intragastric injection of water Ammolipide (Vitrum) and Meritene. They were nursed with great care in order to maintain their weights.

The behaviour of all the operated animals was carefully observed. Special attention was devoted to spontaneous activity and motor ability The animals were regularly tested for catalepsia in the way described by Balagura *et al* (1969) *i.e* the paws were placed one by one on a small box and the time during which the rat kept its paw on the box was registered with a stop watch. Apart from observations of the spontaneous activity of the animals their activity was registered in an Animex activity box (Farad electronics, see Svensson and Thieme 1969) for 15 minute periods every day during the first week after the operation. Some animals of particular interest were registered continously for 24—48 hours. Normal unoperated or saline injected animals were used as controls in the experiments (group 12)

Five animals in group 3 (6-OH DA injection into the substantia nigra) were tested on apomorphine (5 mg/kg) on the third day after the operation shortly before being sacrificed.

In a final series of experiments rats were kept on a 23 1/2 hours water deprivation schedule Food was then always available. The animals were treated with Pimozide which is known to block DA receptors in the central nervous system (Andén *et al* 1970) and apomorphine which stimulates DA receptors (Andén *et al* 1967 Ernst 1967) Pimozide in doses of 1 mg/kg, 2 mg/kg 5 mg/kg and 10 mg/kg was given 4 hours before the drinking period and apomorphine in doses of 0 1 mg/kg 0.2 mg/kg 0.5 mg/kg and 1 mg/kg 3 minutes before. These time periods were selected in order to achieve an optimal drug action at the time of water consumption Pimozide was dissolved in 1—3 droplets of HAc and then diluted in isotonic saline Control animals received saline with added HAc Apomorphine was dissolved in saline All injections were made i.p

The brains from most of the lesioned animals (see below) were analysed with the *histochemical fluorescence method* for monoamines. The animals were decapitated during light chloroforme anaesthesia. The brains were rapidly dissected out, cut in four frontal sections, placed on labeled pieces of stiff paper frozen in liquid propane which was cooled to $-190°$ C by liquid nitrogen. The frozen brain pieces were transferred to a histochemical freeze-drier (FT 1 Bergman and Beving, Stockholm) and processed for 2 1/2—3 1/2 days. The dried pieces were reacted for 1 1/2 hours with gaseous formaldehyd in a closed vessel which was placed in a 80° C oven. The formaldehyde gas was generated from paraformaldehyde powder placed on the bottom of the vessel. The

of axons. There was a strong pile up of fluorescent material in the axons caudal to the lesions (fig. 2) and the corpora striata were devoid of fluorescent DA nerve terminals. There was also a partial disappearance of the fluorescent DA nerve terminals in the olfactory tubercles and the nuclei accumbens due to interruption of the more medially running DA fibres. Parts of the dorso-lateral NA fibres were also lesioned. They constituted however only a minority of the ascending NA fibres. In the 3 animals that did not show adipsia and aphagia the lesions were unilaterally misplaced which resulted in a partial interruption of the nigro-striatal DA fibres and an incomplete DA nerve terminal disappearance from the ipsilateral caudate nucleus.

The animals in group 7 were tube fed in order to maintain their body weights and their behaviour was carefully observed. All 7 animals showed a complete adipsia and aphagia. The animals exhibited varying degrees of motor disturbances. Their general activity was clearly reduced one day after the operation and this state persisted. Three animals were clearly cataleptic, 2 animals weakly cataleptic, while 2 animals showed no catalepsia at all. The catalepsia was evident more or less immediately after the operation. All animals walked with a slightly hunched back and the cataleptic animals tended to spread out their legs—especially the hind legs. The animals actively refused food unless it was placed far back in their mouths. Although catalepsia did not seem correlated directly to the state of adipsia and aphagia the severely cataleptic animals were more difficult to keep alive and 3 of these animals died within 3 weeks. The animals were tube fed for three weeks, during which time the catalepsia gradually disappeared and then dissected and analysed histochemically. The lesions were almost identical to those in the non-supported group of animals. The nigro-striatal DA fibres were completely cut and the corpus striatum devoid of fluorescent DA nerve terminals. There was a considerable decrease in the density of DA nerve terminals in the nuclei accumbens and the tuberculi olfactorii.

Bilateral injection of 6-OH DA into the lateral hypothalamus (group 2 and 8)

The 4 animals in group 2 were intended for histochemical studies and killed 3 days after the operation. At that time they all showed adipsia and aphagia. Their behaviour corresponded closely to that of the tube fed animals (see below). The 6-OH DA was found to be injected in the lateral hypothalamus and there was a strong pile up of fluorescent material in the ascending axons of the nigro-striatal DA system. The majority of the ascending NA axons also showed a strong accumulation of fluorescent material. The corpora striata were

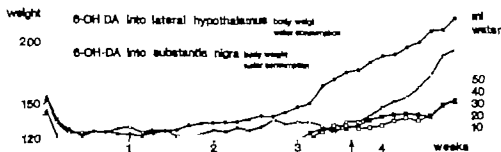


Fig. 3. Water consumption and body weight after bilat ral degeneration of the nigro-striatal DA system induced by 6-OH DA injection t two different sites. Two epresenta tive animals. The arrow indicate when tube-feeding is discontinued.

devoided of fluorescent DA nerve terminals and there was a strong reduction of DA nerve terminals in the nuclei accumbens and the tuberculi olfactorii. At the very tip of the injection cannula there was a small area of unspecific necrosis less than 0.3 mm in diameter The surrounding parenchyma appeared normal except that NA and DA fibres, passing through an area about 1.5 mm in diameter centered around the site of injection, showed a strong pile up of fluorescent material When the 6-OH DA injections were compared to the electrocoagulation of the lateral hypothalamus (group 1 and 7) it was obvious that although the nigro-striatal DA fibres were completely lesioned after both types of lesions, the damage to other neuronal structures than NA and DA axons was far more extensive after coagulation than after injection.

Among the supported animals (group 8) ? animals showed adipsia and aphagia for 2 and 3 days, while the remaining 4 animals showed a severe adipsia and aphagia for 20—28 days (fig 3) They refused food like the animals with lateral hypothalamic electrocoagulations (group 1 and 7) and had to be tube fed. However their motor deficits were much less severe. During the first two days after the operation there was a strong reduction in their normal activity They showed a slight catalepsia and remained akinetic until activated *e g* by handling They walked slowly somewhat hesitatingly with one leg at a time" and a slightly hunched back. The catalepsia quickly disappeared and the akinesia became less pronounced but clearly evident. The animals often remained inactive in their cages in a state reminiscent of reserpinization although they were possible to activate. Two animals developed a slight hyperdipsia after leaving the adipsic and aphagic state. In the final histochemical analysis of the brains of the rats in group 8 the 4 adipsic and aphagic animals showed the same degeneration as the animals in group 2

(see above) and also a clear reduction of NA nerve terminals in the preoptic and septal region. The two animals that showed a very short lasting adipsia and aphagia had remaining DA nerve terminals in the caudate nuclei due to misplacement of the injection cannula

Bilateral electrocoagulation and 6-OH DA injection into the area ventralis tegmenti (group 9 and 10)

Both the electrocoagulations and the 6-OH DA injections lesioned the ascending DA axons where they come together in the rostral area ventralis tegmenti. The corpora striata were devoid of fluorescent DA nerve terminals and there was an almost complete disappearance of DA nerve terminals in the nuclei accumbens and the tuberculi olfactorii. There was also a clear reduction in the number of NA nerve terminals in the hypothalamus and the preoptic area.

All the 8 animals became adipsic and aphagic but the electrocoagulated and the 6-OH DA injected animals differed markedly in their behaviour. The 6-OH DA injected animals behaved very similar to the animals where 6-OH DA was injected into the lateral hypothalamus. They showed 1—2 days of slight catalepsia and then a persistent hypokinesia which decreased when activated e g by handling. Electrocoagulation of the rostral area ventralis tegmenti induced a condition of severe catalepsia where the animals usually remained completely inactive in their cages. The animals were supported for two weeks and then analysed histochemically (see above)

Bilateral injection of 6-OH DA into the substantia nigra (group 3 and 11)

The animals in group 3 were mainly intended for studying the histochemical effect of the 6-OH DA injections. Out of the 18 operated animals 10 developed complete adipsia and aphagia. Five of these were dissected on the 3rd day after the operation for histochemical analysis, while the rest were left unsupported (2 animals died on the 4th day and 3 animals died on the 5th day after the operation). The remaining 8 animals that did not develop complete adipsia and aphagia showed periods of hypodipsia and hypophagia ranging from 1 to 8 days. They were killed on the 9th day

All of the adipsic and aphagic animals showed an increase in their general activity on the first day after the operation (fig 4). The duration of this hyperactivity varied between 1 and 2 days. The hypodipsic animals having the longest period of hypodipsia (8 days) showed a similar but less pronounced increase in general activity. The nature of this hyperactivity is discussed below in connection with the group 11 animals.

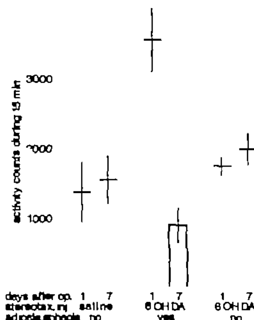


Fig. 4 Activity measured with the Animex one and seven day after the injection of saline or 6-OH DA into the substantia nigra. 6-OH DA animals that de loped aphagia and adipsia showed strongly increased activity one day after th operation and a decreased activity seven days after the operation as compared to saline injected or 6-OH-DA injected animals that showed no adipsia and phagia. The latter h d an incomplete degeneration of the nigro-stri tal DA system.

After the injection of 6-OH DA into the substantia nigra the DA cell bodies showed the typical yellow autofluorescence as well as fragmentation of the pericarya (Ungerstedt 1968 1971b) The extent of the involvement of the DA cell group in the zona compacta of the substantia nigra varied between animals and thus resulted in varying degrees of disappearance of fluorescent nerve terminals from the corresponding corpora striata. The extent of disappearance of DA nerve terminals in the corpora striata was directly proportional to the extent of adipsia and aphagia. The completely adipsic and aphagic animals were practically devoid of DA nerve terminals in the caudate nuclei The hypodipsic animals showed varying degrees of DA innervation in the caudate nuclei. In some animals nerve terminals remained only on one side How ever even the 2 animals showing only one day of hypodipsia and hypophagia had clear reduction in the DA innervation of the corpora striata.

The animals in group 11 were carefully supported by tube feeding from the 2nd day after the operation. Out of 12 animals 6 developed complete

adipsia and aphagia They remained adipsic and aphagic for 24—40 days (fig 3) The remaining 6 animals showed periods of hypodipsia and hypophagia ranging from 1 to 5 days. The tube fed animals (group 11) showed the same behaviour as the non-supported animals (group 3) except for a much better physical condition. All adipsic and aphagic animals as well as 2 hypodipsic and hypophagic animals showed the same increase in their activity for 1—2 days as was noted for the non-supported animals (fig 4) The extent of this increase in activity varied between animals, but showed a maximum between 24—36 hours after the 6-OH DA injection. The animals engaged in a continous motor activity ranging from vigorous running back and forth to automatic walking around in the cages. During these activities they typically showed continous weak sniffing Although the behaviour had this stereotyped character it was clearly influenced by the environment. Sudden sounds elicited virtual motor explosions in some animals. On the second day the activity decreased. In some less active animals the motor activity ceased completely while others engaged in an automatic walking back and forth while maintaining a slightly hunched back and straightened legs. The hyperactivity lasted at the most 1½ day The hypodipsic and hypophagic animals appeared normal after the period of hyperactivity while the adipsic and aphagic animals became strongly hypokinetic (fig 4) When left undisturbed they usually remained in a position reminiscent of a reserpinized animal, *i.e.* the nose close to the floor slightly hunched back, closed eyes and slight pilo-erection. They showed no spontaneous exploratory activity and seldom groomed themselves. However the animals could be activated *e.g.* by knocking on the cage, or by handling Their activity also increased when housed together with another animal. Although hypokinetic the animals showed no catalepsia During the period of tube feeding the animals tended to become less hypokinetic. The animals started to increase in weight quite suddenly and were able to support themselves shortly afterwards. However even at this stage the animals seemed less active to the observer than normal animals.

In the final histochemistry it was obvious that the extent of the degeneration of the nigro-striatal DA system was correlated to the severity of the adipsia and aphagia as was already found in the non-supported animals (group 3)

Bilateral injection of 6-OH DA into the ascending NA bundle caudal to the substantia nigra (group 4)

The animals developed at the most one day of hypodipsia and hypophagia, showed no stereotyped hyperactivity akinesia or catalepsia. The animals were

killed 1—4 weeks after the operation Histochemically the 6-OH DA injections were found to induce a strong pile up of fluorescent material in all ascending NA axons while the DA neurons were unaffected The whole fore brain was almost devoid of fluorescent NA nerve terminals.

Bilateral electrocoagulation and 6-OH DA injection into the rostral hypothalamus (group 5 and 6)

The 6-OH DA injections were aimed for the ascending DA axons to the nucleus accumbens and the tuberculum olfactorium in order to study the effect of a selective disappearance of the DA nerve terminals in these areas. The animals developed at the most two days of hypodipsia and hypophagia, and showed no hyperactivity akinesia or catalepsia. The animals were killed after 2 weeks. The injections were found to cause an almost complete disappearance of the DA nerve terminals in the nucleus accumbens and the tuberculum olfactorium.

Behaviour induced by apomorphine

After apomorphine (5 mg/kg) the 6-HO-DA lesioned, adipsic and aphagic animals showed an initial period of increased motility and sniffing that often changed into licking and biting They soon developed a furious compulsive gnawing which was far more violent than after the same dose of apomorphine administered to a normal animal The operated animals even chewed up" their front paws, bit off their fingers or ate themselves into their abdomen.

Effects of pimozide or apomorphine on drinking

After one week on the 23½ hours deprivation schedule the animals seemed well stabilised in their daily water consumption and the mean consumption during the next 7 days was compared with the consumption on the 8th day when the animals were pretreated with various doses of pimozide or apomorphine. There was a dose dependent reduction in the water consumption following both drugs (fig 5)

Discussion

This study was undertaken for two reasons. Firstly it seemed possible to evaluate the functional importance of the nigro-striatal DA system by selectively removing it with intracerebral injections of 6-OH DA (Ungerstedt 1968 1971b) Secondly it became apparent from the mapping of the mono-

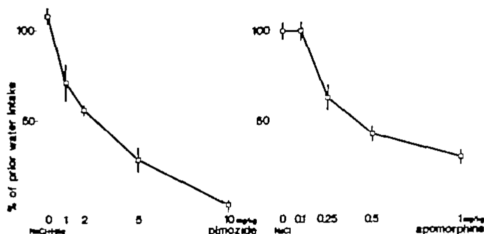


Fig. 5. Effect of pimozide and apomorphine on water intake. The animals were adapted to a 23 1/2 hours water deprivation schedule and the drugs were administered 4 hours and 3 min tes, respectively before th drinking period.

amine pathways (Ungerstedt 1971a) that the DA axons were probably lesioned in a number of previous investigations especially in connection with lesions of the lateral hypothalamus and the symptoms of adipsia and aphagia. Thus, it seemed important to reconsider these studies in the light of the present knowledge.

The experiments in this paper revealed that small electrocoagulations in the lateral hypothalamus interrupt the axons of the nigro-striatal DA pathway (fig 2) and cause the DA nerve terminals in the corpus striatum to degenerate. A number of other neurons are also damaged by the lesions and .g the symptoms of adipsia and aphagia may not be d e to the interruption of DA fibres. However the technique of intracerebral injections of 6-OH DA permits a selective degeneration of DA and NA pathways while there is little damage to other neuron systems (Ungerstedt 1971b) The specific effects of lesioning the nigro-striatal DA system were therefore determined by injections of 6-OH DA into several sites along the path of the nigro-striatal DA system (figs. 1 and 6)

The operated animals were normally divided into two groups. The animals of the first group were killed within a week and served to determine the histochemical effects of th lesions. The second group was kept alive for longer periods of time, while food and water consumption, spontaneous ac tivity motor function and apomorphine induced activity were analysed

When the development of adipsia and aphagia was correlated to the histo-

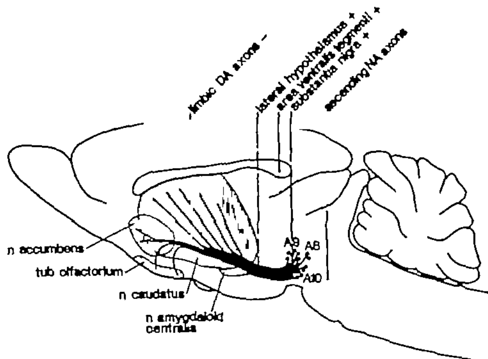


Fig 6 Adipsia and aphagia after 6-OH-DA lesions of the DA pathways. Sagittal outline of the nigro-striatal DA system and the meso-limbic DA system. A8—A10 are DA cell groups in the mecencephalon. A9 is the substantia nigra group. DA nerve terminals are found within the striped areas. Dotted lines indicate the sites of 6-OH-DA injections. + sign indicates that adipsia and aphagia f llowed upon the 6-OH DA injection, while —signs indicat that the injection was ineffective.

chemical effects of the 6-OH DA lesions it was obvious that adipsia and aphagia always followed after a complete bilateral degeneration of the nigro-striatal DA system regardless if 6-OH DA was injected into the substantia nigra the area ventralis tegmenti or the lateral hypothalamus (fig 6) These 6-OH DA injections also damaged parts of the ascending NA pathways as well as the DA axons to the tuberculum olfactorium and nucleus accumbens. However when the ascending NA axons were lesioned by control injections of 6-OH DA behind the substantia nigra, no adipsia and aphagia developed in spite of the fact that most of the NA nerve terminals in the hypothalamus degenerated Lesions of the limbic DA fibres produced at the most a short period of hypodipsia and hypophagia (fig 6)

The extent of the DA denervation seemed critical. A unilaterally incomplete lesion made the animals recover within a few days. The ability to compensate for fairly extensive damage to the nigro-striatal DA system is in all

probability due to the postsynaptic supersensitivity that develops after de nervation of the corpus striatum (Ungerstedt 1971c, 1971d)

The adipsic and aphagic syndrome was extremely severe If not supported the animals died within 4—5 days. Their condition was generally worse than that which is seen when a normal animal is deprived of food and water The animals injected with 6-OH DA into the substantia nigra showed a marked hyperactivity during the first and the second day after the operation The behaviour was reminiscent of that seen after moderate doses of apomorphine. One week after the injection the situation was reversed and adipsic and aphagic animals were clearly hypoactive compared to the animals that did not develop adipsia and aphagia (fig 4) The state of hyperactivity after the injection of 6-OH DA into the substantia nigra may be due to spontaneous release of DA from the degenerating DA nerve terminals in the corpus striatum. This is supported by the fact that the period of hyperactivity ceased at the same time as the DA nerve terminals loose their DA content after an injection of 6-OH DA into the substantia nigra (Ungerstedt 1971 Hökfelt and Unger stedt 1971) Furthermore the period of hyperactivity coincided with the period of spontaneous rotational behaviour in animals with unilateral 6-OH DA le sions which is probably due to a spontaneous release of DA during the nerve degeneration (Ungerstedt 1971e)

During the adipsic and aphagic period, when the animals had to be tube fed all animals showed a pronounced hypokinesia, loss of exploratory behaviour and curiosity and an inability to initiate activity Only when handled or otherwise activated they showed periods of increased activity This condition of hypoactivity was always linked to the adipsia and aphagia regard less of the type of lesion. The condition could be reversed, however by treat ing the animals with the DA receptor stimulating drug apomorphine (Andén *et al* 1967 Ernst 1967) After 5 mg/kg of apomorphine the animals showed increased motor activity that changed into vigorous compulsive sniffing, licking and gnawing The operated animals were more sensitive to apomorphine than normal animals which may be attributed to the supersensitivity of the denervated DA receptor (Ungerstedt 1971c, 1971d) The fact that it was possible to reverse the condition of hypoactivity and induce a state of eating automatism by a DA receptor stimulating agent, supports the hypoth esis that the aphagic, adipsic and hypoactive syndrome is due to the inter ruption of the nigro-striatal DA pathway

It is known that certain neuroleptic drugs, *e.g* pimozide, are highly selec tive blockers of the DA receptors (Andén *et al.* 1970) Such a drug would induce a condition similar to denervation of the DA system. Pimozide did in fact inhibit drinking in animals kept on a deprivation schedule (fig 5) How-

ever the dose dependent inhibition of pimozide may seem inconsistent with the effects of incomplete degeneration of the DA system that did not induce adipsia and aphagia. However pimozide induces a gradually increasing post synaptic block of the DA transmission while function is kept up by the compensatory postsynaptic supersensitivity after incomplete denervation (Ungerstedt 1971c, 1971d)

Although the development of adipsia and aphagia was correlated to the extent of degeneration of the nigro-striatal DA system after all lesions, other behavioural effects varied depending upon where the lesion was situated. A one day period of slight catalepsia was evident after injection of 6-OH DA into the lateral hypothalamus and the area ventralis tegmenti. This was more evident after electrocoagulations when some animals, lesioned in the lateral hypothalamus, showed a catalepsia persistent for about 2 weeks. After electrocoagulations in the rostral area ventralis tegmenti most animals developed serious akinesia, catalepsia and somnolence. Degeneration of the nigro-striatal DA system and the state of adipsia and aphagia were not correlated to these symptoms. All adipsic and aphagic animals, however seemed to be in a state of hypokinesia.

On the basis of the above it seems possible to arrive at some conclusions concerning the behavioural symptoms that may be specifically attributed to the degeneration of the nigro-striatal DA system. None of the animals, where the DA system was degenerated by injections of 6-OH DA, showed any clear cut catalepsia. After the less specific electrocoagulations, however cataleptic states were obvious in several animals. It seems probable that the catalepsia is due to the interruption of some other system than the DA system that passes the lateral hypothalamus and the rostral area ventralis tegmenti. The fact that the 6-OH DA injections into these areas did not produce the state of hyper activity found after injections into the substantia nigra may be due to a disturbance of this system and the animals actually showed a short period of catalepsia. The state of hypokinesia, inability to initiate activity adipsia and aphagia was always associated with each other after a sufficiently extensive bilateral degeneration of the nigro-striatal DA system and is in all probability a result of the degeneration. Although it was possible to support the animals with tube feeding and make them survive until they resumed spontaneous eating 3—5 weeks after the 6-OH DA injections, the severity of the symptoms is obvious from the fact that without tube feeding they died within 4—5 days.

Neither the corpus striatum nor the nigro-striatal DA system has so far been connected with the adipsia and aphagia caused by lateral hypothalamic lesions. These structures are generally thought to participate in motor control and degeneration of the nigro-striatal DA system is thought to be responsible for

the akinesia in Parkinson's disease. The findings in this paper may thus, seem contradictory to the present knowledge. However the existing literature may partly be reinterpreted in the light of the present results to suggest an hypothesis accounting for the observed hypokinesia, adipsia and aphagia as well as the catalepsia.

The nigro-striatal DA system arises from cell bodies in the substantia nigra and terminates in the corpus striatum (for references see Introduction). The course of the pathway has recently been mapped out in detail (Ungerstedt 1971a) (figs. 1 and 6). The axons assemble in the rostral area ventralis tegmenti, ascend in the dorso-lateral part of the medial forebrain bundle and aggregate in a dense bundle in the lateral hypothalamus at the tip of the crus cerebri. The axons then enter the internal capsule, fan out in the globus pallidus and terminate in the corpus striatum, in a dense network of nerve terminals making up about 12 % of the total number of boutons in the nucleus (*Hökfelt and Ungerstedt 1969*). When considering the existing literature it is evident that this pathway has been lesioned in a number of investigations. Von Bechterev (1909) described two dogs with bilateral lesions limited to the substantia nigra. The animals remained curiously immobile despite absence of paralysis or sensory disturbance and did not eat or drink. One dog died on the third postoperative day. Carrea and Mettler (1955), Carpenter and McMaster (1964) and Stern (1966) also found profound hypokinesia in monkeys after lesions of the substantia nigra.

Several authors have studied the effects of bilateral lesions in the region of the area ventralis tegmenti (Ranson and Ingram 1932, Ingram *et al.* 1936, Nauta 1946, Collins 1954). The lesions were consistently associated with adipsia, aphagia, akinesia, catalepsia and somnolence. Catalepsia is defined as a tendency of the animal to remain in any position it is placed in. When the lesions were placed further rostral the hypokinesia remained, while the somnolence and catalepsia was less evident (Nauta 1946, Collins 1954). These results correspond to the findings in the present study after bilateral electrocoagulation of the area ventralis tegmenti.

Lesions in the lateral hypothalamus are commonly associated with adipsia and aphagia and have been described in a vast number of studies since the report of Anand and Brobeck (1951). When the lesion sites are analysed it is obvious that most, if not all lesions, degenerate the ascending DA axons in the lateral hypothalamus. There are comparably few reports in the literature dealing with other aspects of the spontaneous behaviour after such lesions apart from the state of adipsia and aphagia. However, Teitelbaum and Epstein (1962) in their account of the recovery phases of lateral hypothalamic lesioned rats described their initial behaviour as generally inactive but alert and easily

roused to movement. This obviously corresponds to the present findings in the 6-OH DA lesioned rats. Gladfelter and Brobeck (1962) found a permanent decrease in spontaneous locomotor activity after lateral hypothalamic lesions and Beattie *et al* (1969) mentioned a condition approaching catalepsia which could not be reversed by strong tactile stimulation. Balagura *et al.* (1969) described both the state of catalepsia and the state of hypoactivity but found no correlation between catalepsia and deficiency in food and water intake, while hypoactivity in an open field situation decreased after all lateral hypothalamic lesions. Symptoms other than adipsia and aphagia are also apparent after lateral hypothalamic lesions in dogs. Fonberg (1969) and Rozkowska and Fonberg (1970) showed that these lesions abolished "much more than the hunger drive" The dogs lost their interest in the environment spent most of their time lying down, revealed negativism and showed catatonic like symptoms. This syndrome is probably comparable to the lack of exploratory activity and curiosity found after 6-OH DA lesions of the nigro-striatal DA system in the present investigation.

Lesions further rostral in the hypothalamus are reported not to cause adipsia and aphagia (Morgane 1961a) However if the lesions are within the internal capsule or in the globus pallidus they will produce adipsia and aphagia (Morgane 1961a Gold 1967) This is quite striking in view of the anatomy of the nigro-striatal DA system, the axons of which leave the hypothalamus to enter the internal capsule and the globus pallidus. In fact Gold's effective lesions correspond entirely to the nigro-striatal pathway In a recent study Albert *et al* (1970) have made procain injections and tissue cuts in several areas. They concluded that a longitudinal structure including the lateral hypothalamus and the globus pallidus will cause adipsia and aphagia when lesioned Again there is a striking correspondence between their longitudinally oriented structure and the nigro-striatal DA system.

There are areas, however where bilateral lesions produce aphagia and adipsia without interrupting the nigro-striatal DA system *e.g* the mesencephalic reticular formation as described by Gold (1967) Parker and Feldman (1967) and Lyon *et al* (1968) It is also difficult to reconcile the numerous studies on chemical implantations with an effect upon ascending axons of the nigro-striatal DA system (Grossman 1962, 1968 Miller 1965)

When the previous literature and the present results are considered a tentative explanation to the findings may be offered. It is immediately apparent that all experimental observations may not be attributed to a single pathway or center *The catalepsia and somnolence* found after lesions in the region of the area ventralis tegmenti do not depend upon damage to the DA system as selective destruction with 6-OH DA in the same area does not produce these

symptoms. The catalepsia found after lesions of the lateral hypothalamus i not due to a lesion of thes DA system for the same experimental reasons. In an extensive study on catalepsia and somnolence Collins (1954) attributed these symptoms to the interruption of a descending path extending from the caudal hypothalamus to the mid-brain tegmentum terminating at the level of the third nerve. *The hypokinesia the inability to initiate activity and the adipsia and aphagia* however seem closely correlated in several species (see above) *and in view of the present experiments with 6-OH DA these symptoms* may indeed be attributed to the nigro-striatal DA system. *The adipsic and aphagic syndrome arising from the mesencephalic lesions* (see above) has been connected to the interruption of two systems (Blatt and Lyon 1968) The first is a descending pathway from the basal ganglia or the cortex, and the second appears to be one component of an ascending reticular formation pathway The descending pathway may be identical to the pallidofugal pathway that Morgane (1961 1969) found to be interrupted by far lateral hypothalamic lesions. *The eating and drinking elicited by chemical stimulation* is in all probability due to interference with synapses within the hypothalamus. Recent studies using monoaminergic drugs in connection with chemial implants further underline the interpretation that the elicited effects are due to interference with synapses within the hypothalamus and not with a passing fibre system (Booth 1968 Slangen and Miller 1969 Leibowitz 1970) Nor adrenaline nerve terminal areas have been described in detail by Fuxe (1965) using the histochemial fluoresence technique and seem to correspond to the areas where noradrenaline elicits eating In fact, Booth (1967) states that the site where noradrenaline is most effective in eliciting eating is medial to the area where lesions produce adipsia and aphagia.

Existing anatomical studies seem to agree with the above postulated path ways. Guillery (1957) Nauta (1958) and Wolf and Sutin (1966) found one descending fibre system passing from the hypothalamus into the ventromedial tegmentum possibly corresponding to the system described by Collins (1954) in relation to catalepsia, and one system passing into the periaqueductal gray and, thus, possibly connected to the mesencephalic adipsia and aphagia. The third system, the nigro-striatal DA pathway has been mapped in detail (Ungerstedt 1971a) and it represents the only one of these systems whose origin, path and termination is fully known.

The lateral hypothalamic syndrome of adipsia, aphagia and hypokinesia may thus, be attributed to the bilateral interruption of the nigro-striatal DA system and possibly also to the interruption of a pallidofugal fibre system. This remains somewhat uncertain as the lesion described by Morgane (1961a) might have lesioned the DA system as well. However both the DA system and

Kungl. Boktryckeriet P. A. Norstedt & Söner

Stockholm 1971

713093

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 368

DEVELOPMENT OF CATECHOLAMINE-STORING CELLS IN HUMAN FETAL PARAGANGLIA AND ADRENAL MEDULLA

A HISTOCHEMICAL AND ELECTRON MICROSCOPICAL STUDY

BY [1]

ANTTI HERVONEN

HELSINKI 1971

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 368

FROM THE DEPARTMENT OF ANATOMY UNIVERSITY OF HELSINKI
SILTAVUORENPENGER, HELSINKI FINLAND

DEVELOPMENT OF CATECHOLAMINE-STORING CELLS IN HUMAN FETAL PARAGANGLIA AND ADRENAL MEDULLA

A HISTOCHEMICAL AND ELECTRON MICROSCOPICAL STUDY

BY

ANTTI HERVONEN

HELSINKI 1971

Polytypos Turku 1971

FRRATA

Page	Line	
9	13	"Siegrist et l. 1969" read Siegrist et al. 1968
10	17	"Hamilton (1960) please disregard the reference
13	6	"Erlakö (1967) read Erl kö (1967 a, b)
	23	"Enemal, Falck & read Enemar Falck &
14	7	Erlakö (1967) read Erlakö (1967 a, b)
	*9	"Grillo (1960) please disregard the reference
15	1	"Olsson (1970) read Olson & Ungerstedt (1970)
	3	"Olsson (1967) read Olson (1967)
23	32	"Keene & Hewer" please, add t the list f ref rences the f llowing Keene I M. F & Hewer F E.: Obsers tions on the dev lopment f huma suprarenal glands. J Anat. 1927 61 302—324
23	1.	"Matthew read Matthews
26	6	"K fmann (1935) read Kofman (1937)
	7	"Bakay (1937) read Baka (1938)
	14	"Viragh & Korenyi Both (1965) read Viragh & Korenyi Both (1967)
34	18	"Korfman 1935, Bara 1937" read K fma 1937 Bakay 1938
44	*7	"I anow 1034 read I anow 1932
45	14	"Roffi 1968" read Roffi 1968 a, b
46	2	"Coupland 1963 a, b read Coupland 1963
55	11	longated" read elongated
69	5	"radely" read rarely
71	19	"Elfvin (1966) read Elfvin (1963)
73	23	"Siegrist et l. 1966 1968 read Siegrist et al. 1968
73	14	"Viragh et l. (1966) read Viragh & Korenyi Both (1967)
76	20	"Tennyso (1969) read Tennyson (1970)
79	35	"Grillo (1964) please disregard the reference
	22	"Villee (1969) please disregard the reference
80	12	Korochkin & Korochkin 1969 read Korochkin & Korochkina 1970
83	6	"Ehinger t al. 1967" read Ehinger et l. 1968
88	19	"Champlan, J de Malfora, T read Champlain, J de Malmfors T
90	34	"Geanner" read Geneser
94	10	"Viragh Sz. & Korenyi Boh A read Viragh, Sz. & Korenyi Both, A.

ACKNOWLEDGEMENTS

It is pleasure to express my deep gratitude to Professor Olavi Eränkö M.D., Ph.D., who proposed the subject of this study to me for the first time on 21 1.1966 He has since patiently guided my studies, proceeding with variable efficiency to the present stage Being allowed to work in the Department of Anatomy of University of Helsinki headed by him was of decisive importance during the critical stages of the work.

I have always found the atmosphere of the department pleasant and stimulating for my scientific efforts. For this I owe gratitude to all members of the staff of our laboratory

I am particularly indebted to my friend and fellow investigator Lasse Kanerva M.L., for his continuous encouragement during these studies. The instructive examples set by Dr Leena Rechardt, M.D and Professor Arto Palkama, M.D and the innumerable inspiring discussions with these colleagues were of the greatest importance and I am most grateful to them

Very special thanks are also due to Mrs. Liisa Kangasvuo Mrs. Pirkko Alanko and Miss Raisa Männikkö, for their skillful technical assistance. Further I should like to thank Miss Helmi Palomäki, Librarian of the Department, for help in obtaining the litterature and Miss Irma Enrold for typing the manuscript.

I am grateful to Mr Mauri Nyholm, M.A. and the staff at the Department of Electron Microscopy University of Helsinki where the electron microscopic studies were started.

I wish to thank Professor Erkki Järmori, M.D the head of the College of Midwifery Helsinki, Finland, who allowed me to collect the material in his hospital. I am also grateful to Miss Irja Sulander and the staff of the operating theatre for their valuable practical help

My best thanks are due to my wife Pirkko and our children, Johanna and Petteri you provided an energy releasing stimulus by entering my life.

Helsinki 23 4. 1971

AH

ffin cell refers to a cell which shows an intense yellow immersion in aqueous dichromate solution. Although lly used in relation to a certain histological method, ffin tissue has become established. The para-aortic lulla are classic examples of true chromaffin tissues n 1903 Coupland 1952)

f the true chromaffin cell has been elucidated by see Chapter IV) and the most prominent cyto- cell is the presence of large amounts of typical les. In recent electron microscopic papers the has been widely used as a general name for cells mbles the well-known characteristics of true t al 1969 Mascorro & Yates 1970 Jacobowitz shown, however that there are cells which are ntical with the true chromaffin cells, but do maffin reaction (Eränkö and Härkönen 1965 Palay 1969 Matthews & Raisman 1969 Eränkö s was pointed out by Williams & Palay (1969) annot properly be used in studies which do n.

term *CA storing cell* was used when the lionic and the adrenomedullary cells was n l In the electron microscopical part of the *g cell* was used, instead of names referring ntent.

malian species are known to possess abundant extra-adrenal chromaffin tissue rabbit and man (Coupland 1965) Hence the human fetus serves as an excellent object for studying the development and nature of maturing chromaffin tissue.

Hypotheses concerning the functional nature of paraganglia and fetal CA-storing tissue in general have been presented by several authors (for ref. see Coupland 1965) based on investigations performed with mammalian material. The main questions left open by these studies were listed as follows

— What are the characteristics of differentiation of CA-storing cells of human fetal paraganglia and adrenal medulla?

— When does CA-synthesis start in developing CA-storing cells?

— Does the fetal CA-storing cells posses a functional innervation?

— How are the fetal CA-storing cells related to the microcirculation with special consideration of possible endocrine function?

TERMINOLOGY

The term chromaffin cell refers to a cell which shows an intense yellow brown reaction after immersion in aqueous dichromate solution. Although the name was originally used in relation to a certain histological method, the concept of "chromaffin tissue" has become established. The para-aortic bodies and adrenal medulla are classic examples of true chromaffin tissues (Zuckerkandl 1901 Kohn 1903 Coupland 1952)

The fine structure of the true chromaffin cell has been elucidated by several authors (for ref see Chapter IV) and the most prominent cytoplasmic feature of the cell is the presence of large amounts of typical catecholamine-storing vesicles. In recent electron microscopic papers the term "chromaffin cell" has been widely used as a general name for cells whose ultrastructure resembles the well-known characteristics of true chromaffin cells (Siegrist *et al* 1969 Mascorro & Yates 1970 Jacobowitz 1970) It has been clearly shown however that there are cells which are electron microscopically identical with the true chromaffin cells, but do not exhibit a positive chromaffin reaction (Eränkö and Härkönen 1965 Norberg 1965 Williams & Palay 1969 Matthews & Raisman 1969 Eränkö & Eränkö 1971) Therefore, as was pointed out by Williams & Palay (1969) the term "chromaffin cell" cannot properly be used in studies which do not involve a chromaffin reaction

In the present study the term *CA storing cell* was used when the development of the paraganglionic and the adrenomedullary cells was studied at light microscopical level In the electron microscopical part of the study the term *granule-containing cell* was used, instead of names referring to the chromaffinity or amine content.

MATERIAL

The present material was collected between 1967 and 1970. All fetuses were obtained from legal interruptions of pregnancy performed by laparotomy (*sectio minor*). In connection with the operation the patients received the following drugs. As premedication before the operation pethidin cloride (Pethidin®) 1 mg/kg, promethazine cloride (Phenergan®) 0.7 mg/kg and atropin 0.5—1.0 mg. Thiomebumal sodium (Intraval Natrium®) was given intravenously for induction of anesthesia, which was continued with nitrous oxide. Succamethone chloride (Scoline®) infusion was used for muscular relaxation. No other drugs, including psychopharmacological agents, were used.

The age distribution and the use of the total material is listed in Table I.

There is no absolutely accurate method of estimating the age of an embryo from its length or weight because of the considerable variations from one embryo to another. The estimation of gestational age was made according to statistics summarized from the measurements of Scammon and Calcins (1929), Streeter (1948) and Hamilton (1960).

With special arrangements in the operating theatre the processing of the fetuses could be started within 1—2 min. of the disconnection of feto-placental circulation. Only fetuses which could be processed immediately were accepted for fluorescence microscopy demonstration of cholinesterases and electron microscopy. Older fetuses were also used for angiography and routine light microscopic specimens.

No	CR	FA (weeks)	AChE/ChE	FIF	EM	AGR	LM	No	CR	FA (weeks)	AChE/ChE	FIF	EM	AGR	LM
1	1.2	5,6			X			51	8.3	13,7		X			
	1.4	5,9		X	X			52	8.4	13,8				X	
3	2.0	6,7	X		X			53	8.5	14,0				X	
4	[illegible].1	6,9				X		54	8.5	14,0			X		
5	2.4	7,3		X	X			55	8.6	14,0				X	
6	2.9	8,0			X	X		56	8.6	14,0					X
7	3.0	8,3		X				57	8.7	14 1					X
8	3.2	8,6	X		X			58	8.8	14,[illegible]					X
9	3.3	8,7				X		59	8.8	14,[illegible]	X				
10	3.5	8,9	X					60	8.9	14,2		X			
11	3.6	9,0		X	X			61	9.0	14,3		X	X		
12	3.7	9,2						62	9.0	14,3		X			
13	4.0	9 7		X	X			63	9 0	14,3		X	X		
14	4.1	9,8	X					64	9 1	14,3					X
15	4.3	10,0				X		65	9.[illegible]	14,4				X	
16	4.4	10,1				X		66	9.2	14,4					X
17	4.5	10,2			X			67	9.3	14 4		X			
18	4.5	10,[illegible]		X	X			68	9 4	14,5		X			
19	4.6	10 4			X			69	9 4	14,5	X				
20	4.6	10,4					X	70	9 4	14,5				X	X
21	5.0	10,8	X		X			71	9 4	14,6		X		X	
22	5.2	11,0			X			72	9.4	14,5					X
23	5 4	11,3				X		73	9.4	14,5					X
24	5.5	11 4					X	74	9.5	14,6					X
25	5.5	11 4		X				75	9.5	14,6				X	
26	5.0	11 7					X	76	9.5	14 6			X		
27	6.0	11,8		X	X			77	9.5	14,6		X			
28	6.0	11,8	X					78	9.7	14,8			X		
29	6.3	12,1					X	79	9 7	14,9			X		
30	6 4	12,2					X	80	9.8	15,0					X
31	6.5	12,3				X		81	9.8	15,0					X
32	6.8	1[illegible],3					X	82	9.8	15,0	X				
33	7.0	1[illegible],7	X	X				83	9.8	15,0	X				
34	[illegible].0	12,7			X			84	9.9	15,0	X				
35	7 1	12,8		X	X			85	9.9	15 0		X			
36	7 1	1[illegible],8		X	X			86	9.9	15,0		X			
37	7.3	13,0					X	87	10.0	15,0			X		
38	7.3	13,0		X	X			88	10.0	15,0	X	X	X		
39	7.5	13,2					X	89	10.0	15,0				X	
40	7.5	13,2				X		90	10.0	15,0		X			
41	7.6	13,2	X					91	10.0	15,0	X	X	X		
42	7.6	13,2				X		92	10.0	15,0		X			
43	7 7	13,4					X	93	10.2	15,[illegible]		X	X		
44	7 7	13,4		X			X	94	10.2	15,3		X			
45	7.9	13,4				X		95	10.3	15,4		X	X		
46	7.9	13,4			X			96	10.3	15,4			X		
47	8.0	13,5		X				97	10.3	15,4		X			
48	8.0	13,5	X					98	10.4	15,4	X		X		
49	8.3	13,7		X	X			99	10.5	15,5			X		
50	8.[illegible]	13,7			X			100	10.5	15,5		X	X		

Table I

Age distribution and use of the material. Symbols CR = Crown-rumpf length, AChE/ChE = Acetylcholinesterase and nonspecific cholinesterase FIF = Formaldehyde induced fluorescence EM = Electron microscopy AGR = microangiography or indian ink technique LM = Control light microscopy

No	CR	age (weeks)	AChE/ChE	FIF	EM	AGR	LM	No	CR	age (weeks)	AChE/ChE	FIF	EM	AGR	LM
101	10.5	15,5			X			151	13.1	1 ,6		X	X		
102	10.5	13,5	X					15_	13_	17 6		X			
103	10.5	15,5	X		X			153	13.2	17 6					X
104	10.	15,7					X	154	13.4	17,8			X		
105	10 7	15,7	X					155	13 4	17,8		X			
106	10	15 7		X				156	13.5	17,9					X
107	10.9	16,0	X	X		X		157	13.5	17,9			X		
108	10.9	16,0					X	158	13.5	17,9	X				
109	10.9	16,0					X	159	13.5	17,9					X
110	11.0	16,0			X		X	160	13.5	17,9	X				
111	11 0	16,0	X	X	X			161	13.8	18,1	X			X	
112	11.2	16,3	X		X			162	13.8	18,1				X	
113	11.3	16,2	X					163	13.9	18,3	X	X			
114	11.3	16,2	X					164	14.0	18,4					X
115	11.3	16,_					X	165	14.0	18,4	X				
116	11.3	16,2	X	X	X			166	14.1	18,4			X		
117	11.4	16,4		X				167	14.3	18,7		X	X		
118	11 4	16,4		X				168	14.5	18,8				X	
119	11.5	16,4					X	169	14.5	18,8				X	
120	11.6	16,3					X	170	14 7	18,8			X		
1 1	11.6	16,5				X		171	14.8	19,0				X	
122	11.6	16,5					X	172	14.8	19 0				X	
123	11.6	16,5					X	173	14.9	19 1		X			
1 4	11.8	16,6					X	174	14.9	19 1				X	
125	11.9	16,7					X	175	14.9	19 1	X				
126	11.9	16,7	X					176	15.0	19,_			X		
12	11.9	16,7	X					177	15.0	19,3	X				
128	1_0	16,8			X			178	15.1	19,3	X				
129	1_0	16,8	X					179	15.1	19,3		X			
130	12.0	16,8	X	X	X			180	15.1	19,3			X		
131	1_0	16,8			X			181	15.2	19 4		X			
13_	12.2	17,0		X				182	15.5	19,6		X	X		
133	1__	1 ,0		X				183	15.6	19 6	X	X	X		
134	12.2	1 ,0		X	X			184	15.6	19,8	X				
135	1__	17,0				X		185	16.0	20,2			X		
136	1_4	1 ,0				X		186	16.2	20 4	X	X	X	X	
137	1_4	17,0				X		187	16.7	_0,8					X
138	1_6	17,2	X					188	16.7	20,8	X		X		
139	1_7	17,3	X					189	17 1	21 1			X		
140	12.7	1 ,3				X		190	17 4	21 4		X			
141	12.	17,3	X	X	X			191	17.5	21,3	X	X	X		
14	1_9	17,5		X				19_	17 7	_1,6	X		X		X
143	12.9	1 ,3		X				193	18.0	22,0			X		
144	1_0	17,3	X	X				194	18.2	22,2	X	X			
145	1_9	1 5	X					195	18.5	22,5				X	
146	13.0	17,5	X					196	19._	23,1			X		
147	13.0	17 5		X				197	18 7	23,8	X				X
148	13 0	17,5		X	X			198	1.5	4,1	X	X	X		
149	13.1	17,6	X	X				199	22.7	26,7	X	X	X		
150	13 1	17,6		X	X			200	23.0	_7,0	X	X	X		

Table I

Age distribution and use of the material. Symbols: CR = Crown rumpf length, AChE/ChF = Acetylcholinesterase and nonspecific cholinesterase, FIF = Formaldehyde induced fluorescence, EM = Electron microscopy, AGR = microangiograph or Indian ink technique, LM = Control light microscopy

CHAPTER I

MONOAMINES DEMONSTRABLE WITH FORMALDEHYDE INDUCED FLUORESCENCE IN PARAGANGLIA AND ADRENAL MEDULLA

Introduction

After the first demonstration of formaldehyde-induced fluorescence by Eränkö (1951 1952, 1955) a basic method for localization of catecholamines was available whose specificity had been checked by chemical analysis of microdissected pieces of adrenal medulla. Several later modifications of the method increased localization power (Eränkö 1961 Falck & Torp 1961 Falck *et al* 1962, Eränkö 1964 Falck & Owman 1965 Eränkö 1967) The mechanism of the reaction between catecholamines and formaldehyde has been studied in particular by Corrodi & Hillarp (1963 1964) Corrodi and Jonsson (1965 a & b, 1967) and Jonsson (1967 a & b) Thus, to date the demonstration of FIF serves as a reasonably reliable and specific histochemical method for catecholamines, especially when minimal amounts of them are present in the tissues studied

The catecholamine content of human fetal paraganglia and adrenal medulla has been studied by using both the chromaffin reaction and pharmacological estimations of catecholamines. Coupland (1965) stated that no positive chromaffin reaction has been observed prior to the 55 mm (11 0 weeks) stage although the developing chromaffin cells could be identified even after the 27—30 mm (c. 8 weeks) stage by their nuclear morphology and topographical position. It is generally accepted however that the chromaffin reaction is rather insensitive and unspecific for demonstration of smaller amounts of catecholamines. Studies on the FIF in developing sympathetic nervous system and chromaffin tissue have recently been published (Enemar Falck & Håkanson 1965 Björklund, Enemar & Falck 1968 Champlain *et al* 1970) All these authors found a relatively early appearance of FIF in the developing chromaffin system of chick, rat and mouse embryo They further emphasized the value of the present method in detecting the appearance of the first catecholamines during the development. Apart from a preliminary report by the present

writer (Hervonen 1970b) no studies on the FIF of human fetal paraganglia and adrenal medulla were available

Because of the specificity of the present method (see Corrodi & Jonsson 1967) there is no doubt that the FIF observed is due to an aromatic monoamine For discrimination between different amines, noradrenaline adrenaline and dopamine however other means must be employed (see Eränkö 1967)

Several attempts have been made to estimate the quantity and quality of catecholamines in human fetal chromaffin tissue pharmacologically and biochemically Hunter *et al* (1953) and Niemineva & Pekkarinen (1953) observed that the amounts of catecholamines extracted from the extra adrenal chromaffin tissue exceeded those found in the adrenal glands of the same human fetuses. Similar quantitative results have been reported by several authors (for ref see West *et al* 1953) Separate determinations of noradrenaline and adrenaline showed that the pressor substances in human fetal chromaffin tissue consisted almost exclusively of noradrenaline (for ref see Brundin 1966) Von Studnitz (1965) determined the catecholamine content of midterm human fetal adrenal glands and found considerable amounts of both NA and A Recently Genneser & Studnitz (1969) also found traces of adrenaline in the paraganglia of midterm human fetuses. Then enzyme phenylethanolamine-N-methyl trasferase essential for methylation of noradrenaline to adrenaline was also observed to be functional in fetal paraganglia.

The observation of the small intensely fluorescent cells" (SIF) in the sympathetic ganglion of the rat (Eränkö & Härkönen 1963) aroused a new wave of interest in the CA-containing cells situated extra-adrenally These "SIF-cells" gave a negative chromaffin reaction (Eränkö & Härkonen 196 Norberg *et al* 1966) although their fine structure closely resembled that of chromaffin cells (Eränkö & Härkönen 1965 Grillo 1966 Elfvin 1968 Siegrist *et al* 1968, Matthews & Raisman 1969) It is probable that the SIF-cells also originate from the primitive sympathetic cells. True chromaffin cells are found, according to Coupland (19 _) in all parts of the prevertebral sympathetic plexus of abdomen and pelvis and, less commonly in association with the sympathetic chain in the human fetus. The same author found separate collections of chromaffin cells within the sympathetic ganglia in the prevertebral plexus. No other reports on the presence of chromaffin cells (or SIF cells) in human sympathetic nervous tissue were available The distribution of developing CA-storing cells within the preaortic sympathetic nervous tissue was therefore given particular attention

Methods

The general rules presented by Frankö (1967) and Olsson (1970) have been followed. It is difficult to freeze-dry embryological material, possibly due to its high water content (see Björklund *et al.* 1968, Olsson 1967). Several combinations of the basic parameters influencing the final result of freeze drying were therefore compared to find the most suitable conditions for human CA-storing cells. The drying temperature varied from —50 — —30° and the vacuum between 10^{-4}—10^{-5} mmHg. The drying procedure lasted for 1—6 days. For human fetal tissue drying temperature of —35°C and drying time of 2—3 days proved most suitable.

The exposure to paraformaldehyde vapour was performed at 60—80 °C for 15—60 min. To detect secondary amines, longer exposures up to 180 min were also used. The relative humidity of the paraformaldehyde vapour varied mostly between 60—80 %.

The adrenal glands and main para-aortic bodies were prepared within 1—2 min. of disconnection of the feto-placental circulation. Thin slices of the medullary area of the adrenals or in smaller fetuses, the whole retroperitoneal block containing both adrenals and the preaortic area were cut and immersed in propane or isopentane cooled in liquid nitrogen. The frozen tissue pieces were either stored in liquid nitrogen or immediately processed further.

A combination of mechanic and diffusion pump as described by Frankö (1967) or an efficient mechanic pump alone were used for drying. A chemical water trap was used in the form of large amounts of P_2O_5.

After the drying procedure the desiccators were warmed up to 50—60°C and the specimen holder rapidly moved to the glass jar containing formaldehyde powder. The formaldehyde-treated tissue pieces were embedded in paraffin or for better resolution in epoxy resin. The following mixture of epoxy resin was used: Araldite 502, 15 ml, Epon 812, 25 ml, dibutyl phtalate 4 ml, 2.9 ml of dodecinyl succinic anhydride and 8 drops of benzyl dimethylamine was added to this mixture (Fränkö & Fränkö 1971). 1—5 micron thick sections were cut from the epoxy-embedded specimens with an LKB-pyramitome using a glass knife. Mainly Wild's monocular microscope was used for fluorescence studies. The light source was a high pressure mercury lamp HBO 200 (Osram). The Schott BG 38 was used as a heat absorbing filter, BG 12 as blue filter and the OG 1 as the secondary filter. Toluidine blue control stainings were made after the fluorescence microscopy.

adrenal cortex was found here the tight organization of early CA-storing cells was broken up and the cells were dispersed amongst the cortical tissue (Fig 8)

Fluorescent cells were found intra-adrenally within a rather restricted area consisting of a invasion zone on the medial side of the gland Both primitive sympathetic cells and fluorescent cells were also found in the central region of the cortical mass. Solitary cells or small groups of cells were scattered evenly through the central areas and the invasion route connecting extra-adrenal fluorescing elements with the premedullary area. (Fig 9)

A intensification of FIF of both extra and intra-adrenal collections of fluorescing cells was observed during the 10th week of development. The proportion of yellow FIF increased and at the end of the 11th week the great majority of cells were exhibiting a fluorescence of moderate intensity which was yellow in c. 60 % of the cells (Fig 3) The amounts of fluorescent cells in both paraganglia and adrenal medulla also increased rapidly and at the end 12th week the organization of the CA-storing cells was the following

Only brightly yellow fluorescent cells could be found within the large developing para-aortic bodies. The cellular organization was regular: cords or columns of cells in close contact with capillaries were found (Fig 2 and 5) The total mass of abdominal para-aortic bodies was greatly increased from the age of 9 weeks. At this stage FIF was also observed in the cytoplasm of the developing sympathetic neurons in ganglion coeliacum closely associated with the paraganglia. In addition to the main para aortic bodies, large numbers of small r clusters of brightly yellow fluorescing cells were found along the preaortic sympathetic plexus. These clusters were frequently met after this age within the boundaries of devel oping coeliac ganglion as well

The number of fluorescent cells within the adrenal cortical area was clearly increased even considering the simultaneous condensation of the

Fig 7 Weakl fluoresci g cells on the interadrenal area f 8 weeks-old fetus. The lrenal cortex in the right lower corner f the figure Fluorescing cells could not be observed within the boundaries f th cortical elements. (AC=adrenal cortex.) 225 ×
Fig 8 The central region of the drenal gland of 9 weeks ld-fetus. Solitary fluoresci g cells could be found scattered evenl through the cortical matrix. 1 0 ×
Fig 9 A irenomedull ry CA-storing cells of a 11 weeks old fet s. Onl small groups could be found. (AC =adrenal cortex.) 225 ×
Fig 10 The medullary cells in drenals f 1 weeks ld fetus. The cells form cords and colum I addition t the mall ovoid groups. 340 ×
Fig 11 The group f primitive sympathetic cells urrounded by med llary CA-storing cells. Not the gra larity f the fluorescence Section thickness 1 micron. (PSC=primi tiv sympathetic cells.) 670 ×

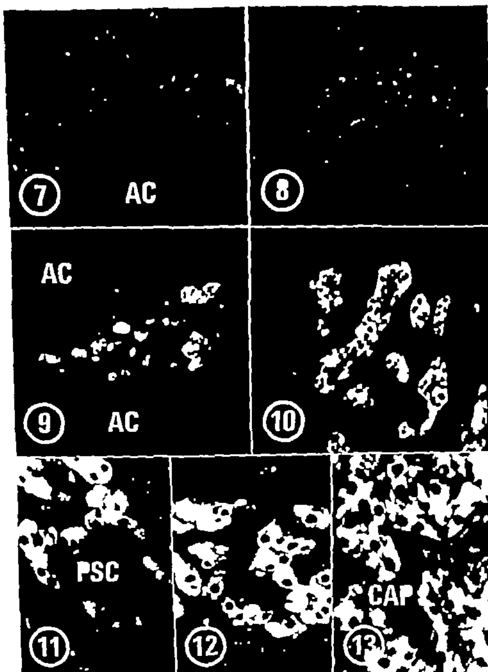


Fig 12 Cords of medullary cells i the central region f adrenals of 18 week old fetus. Also here the internal gran larity of the FIF is clearly isible. 670 ×
Fig 13 Paraganglionic CA-storing cells from the main para-aortic body of 20 weeks *old-human fetus. The cells are similar to the adrenomed llary CA-storing cells. The color* of the FIF is brightly yellow (CAP=Capillary) 670 ×

medullary elements. Thus, the relative density of fluorescent cells in the central part of the fetal cortex was multiplied. The former grouping of fluorescent cells (Fig 14) in small groups was partly replaced by chains and cords or elongated forms of cell collections (Fig 10) The fluorescent cells were in intimate contact with the capillary sinusoids and the fetal cortical cells.

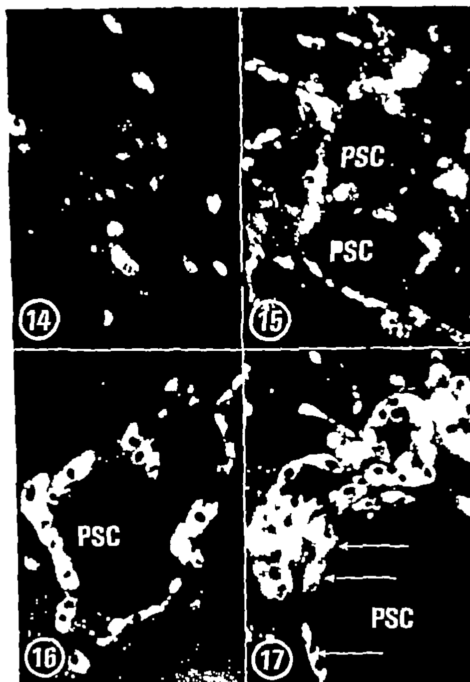
The distribution of primitive sympathetic cells in the preaortic area and within the adrenal cortical elements was studied from the toluidine blue stainings and also from the series prepared for circulatory studies (Indian ink injections) Large groups of primitive sympathetic cells were found mainly on the central region of the fetal cortex (Fig 14) During the third month, the majority of these groups also gathered in the premedullary area. After the eleventh week, green FIF could be observed in some cells on the marginal zone of the collections of primitive sympathetic cells and a typical organization of fluorescing cells around the groups soon developed. After the 12th week brightly fluorescing cells were regularly found to surround the groups of primitive sympathetic cells (Fig 15—17) These newly differentiated fluorescing cells were called "intracapsular cells because they were situated inside the thin connective tissue capsule surrounding the groups of primitive sympathetic cells (Fig 17) A weak green FIF appeared in several cells within the groups of primitive sympathetic cells, but the FIF in this localization always remained very weak. The CA storing cells were always in close contact with the wide capillary sinusoids of the premedullary area or with the cells of the fetal cortex.

At the end of the third month, only fluorescent cells could be observed in the paraganglia and no primitive sympathetic cells or other non fluorescent cells could be found during the later stages. Instead, intra-adrenally large amounts of non fluorescent primitive sympathetic elements persisted throughout the period studied.

While the essential characteristics in the development of CA-storing cells during the first trimester of pregnancy the organogenesis period,

—

Fig 14 A overexposed photograph from the adrenal med lla f 11 weeks old fetus t how the collections f primith sympathetic lements. The cortical cells exhibit moderat at fluorescence (arrows= group f primitive sympathetic cells.) 3×0 ×
Fig 15 Two dreamomedullary collection f primiti ympathetic lements surrounded by rim f tea ly fluorescing maturing medullary cells. A 1 weeks old fetus. (PSC primith sympathetic cells.) 3×0 ×
F 1 A group f primith sympathetic cells in adrenals f 16 weeks old fetus. Th medullary A oring cells surround the primitive lements. (PSC=primitiv sympathetl cells 6?
Fig 1 A wood large collection f drenal primitive sympathetic cells. The sur-



rounding mature medullary cells were seen in two positions outside the connective tissue capsul and inside it. A weak greenish FIF could be seen in some cells of the group of primitive sympathetic cells. A 16 weeks old fetus. (PSC=primitive sympathetic cells, arrows=intracapsular medullary cells.) 700 ×

were concerned with the appearance intensification and spreading of FIF through the preaortic area and adrenal glands, only minor changes in the intensity of FIF or in the localization of CA-storing cells were observed after the 14th week of fetal age. The estimated total amount of both extra adrenal and intra-adrenal fluorescent tissue increased evenly during the following three months as well.

The FIF in developing CA-storing cells was usually homogenous before the fourth month, and no substructures could be found within the cytoplasm. However after this age when the 1 micron sections were used, a clear granular substructure could be demonstrated after initial slight fading of the fluorescence intensity (Fig 11 and 12) If properly prepared specimens were used this substructure could regularly be found in both extra and intra-adrenal fluorescing cells. All cells of the paraganglia were fluorescing highly yellow and only minor differences between individual cells could be seen after the age of 14 weeks To visualize better the relationships of the amine storing cells to the capillaries, indian ink injections were given to three of the fetuses prior to the freeze drying (for details of the method see Winckler 1969) An intimate contact between capillaries and the paraganglionic cell-cords was demonstrated in all specimens. The proportion of capillaries from the cross-sectional area gradually increased, covering c 30 % of the total area at the 20th week (Fig 6) The whorled organisation of cells in the paraganglia became clearer (Fig 4 and 6 with increasing age)

As a result of the condensation of CA-storing and primitive sympathetic elements on the central region of the cortical tissue a clear medullary group of cell clusters was formed. The boundaries towards the cortex were obscure because the groups of fluorescing cells were still scattered among the anastomosing cords of cortical cells. With increasing age however the proportion of cortical cells decreased in the premedullary area. The final organization of the fluorescing cells in adrenal medulla was the following (a) The cells formed rounded or ovoid groups or were organized in whorls. The cells were mostly on the wide capillary sinusoids, and showed tight contact with the capillary wall In thin sections the fine granularity of the cytoplasm could be demonstrated (Fig. 11 and 12) The medullary cells of the over 14 weeks old fetuses were frequently gathered around the collections of primitive sympathetic cells (Fig 17) The intracapsular CA-storing cells" were regularly found forming a surrounding rim around these groups (Fig 15—17) and whorls of CA storing-cells were often found contacting the primitive sympathetic cell groups (Fig 14) (b) Primitive sympathetic elements. Weak greenish FIF

could be found in the great majority of cells inside the groups of primitive sympathetic cells. Intensively yellow fluorescing cells were never found in the central parts of the collections.

A few small clusters of strongly fluorescent cells were regularly found in the preaortic sympathetic ganglions of the older fetuses. Capillaries were usually found in the immediate vicinity of these cell groups.

Discussion

Differentiation of primitive sympathetic cells

Fluorescing cells were found for the first time within the preaortic collections of primitive sympathetic cells of a 7 weeks old fetus. In the earlier reports on the appearance of CA-storing cells in developing human fetus, the monoamines have been demonstrated using the chromaffin reaction, and positivity has not been obtained before the 55 mm stage (11 weeks) (Coupland 1965). Very early appearance of FIF in the chromaffin and sympathetic elements of rat fetus was reported in the recent work of Champlain *et al.* (1970). The changes in nuclear morphology of developing primitive sympathetic cells occurred at the age of 8 weeks, according to Coupland. This finding was confirmed on the basis of the toluidine blue specimens from the Epon embedded tissues. Thus, FIF was demonstrated before any light microscopic changes in the primitive sympathetic cells could be demonstrated, indicating very early presence of CA. This first FIF was also demonstrated after a short exposure to formaldehyde. This and the green color of the FIF indicated the presence of a primary amine, probably noradrenaline.

The further differentiation of primitive sympathetic cells seemed to begin in the collections located in the preaortic area, and the spread of FIF occurred towards the adrenals. However, the induction for the start of the catecholamine synthesis probably proceeded simultaneously in the large preaortic area. The cells invading the cortical elements in smaller groups had probably started differentiation on the "CA-stogare-line" even before the invasion period. Instead, the large collections of primitive sympathetic cells migrating to the centre of cortical elements apparently showed a delayed rate of differentiation. The appearance of intracapsular chromaffin cells has not been described (Keene & Hewer 1927, Coupland 1965). The primitive sympathetic cells persist in the human adrenal gland up to full term and disappear during the first three postnatal years

(Coupland 1965) The present findings showed that the large primitive sympathetic cells groups formed patterns of delayed differentiation from which new adrenomedullary cells differentiated after the 12th week of fetal age

The weak green FIF exhibited by the cells within collections of primitive sympathetic elements indicated that these cells have also started their differentiation The adrenal primitive sympathetic elements thus probably represented a cell population with ability to synthesize or store slight amounts of CA, but were still indifferent with respect to the two alternative differentiational pathways towards CA-storing cell or towards sympathetic neurons.

The increase of CA content in the amine storing cells in the preaortic area was rapid after the 8th week, reaching the fluorescence maximum at the age of 12 weeks. The change in the color of FIF from green to yellow was apparently caused by the color-shifting effect of an increase in the fluorescence intensity without any actual changes in the emission quality (Eränkö 1967) The catecholamine responsible for the yellow FIF is probably noradrenaline (Coupland 1953 Hunter *et al* 1953 Niemineva *et al.* 1953 Gennser *et al* 1969) The FIF was always demonstrable after a short exposure to paraformaldehyde and no additional FIF was found after the prolonged exposure required for the secondary amines such as adrenaline Parallel with the increase of intensity of FIF in the para ganglia, solitary small clusters of fluorescing cells also appeared along the course of the developing sympathetic plexus and were found in close contact with the coeliac ganglion. This is compatible with the earlier observations of Coupland (1952) and Muscholl & Vogt (1964)

The simultaneous differentiation and identical fluorescence characteristics of the brightly fluorescing cells within the abdominal sympathetic ganglia and in the superior cervical ganglion of human fetus (Hervonen Franko & Frankö unpublished observation) suggests that they might be identical to the other CA-storing cells. Hence the "SIF"-cells presumably originate also from the primitive sympathetic cells of the preaortic area.

Relations of fluorescent cells to circulation

The freeze drying method preserves the vascular structures excellently thus affording the possibility of estimating the relations of the CA-storing cells to the circulation The earliest stage of vascular development within the primitive sympathetic elements is studied in Chapter III

The highly fluorescent cells in paraganglia were mostly in intimate contact with blood sinusoids or capillaries, and a very rich capillary network was found at all stages studied. These observations are in agreement with the reports from the early works of Zuckerkandl (1901) and Kohn (1903) and, more recently Coupland (1952). Winckler (1969) found a close association between the chromaffin cells and capillaries in paraganglia of rat. If smaller clusters of brightly fluorescing cells were found along the course of primitive sympathetic plexus or within the coeliac ganglion, capillaries were also present in the immediate vicinity. If larger collections were at hand, the typical sinusoidal capillary supply could always be demonstrated. The finding is in agreement with the results of Eränkö & Härkönen (1965), Matthews & Raisman (1969) and Eränkö & Eränkö (1971), who found the "SIF"-cells of rat sympathetic ganglion in close contact with the capillaries.

In the developing adrenal medulla, the medullary cells were also found on the widened medullary sinusoids. The "intracapsular" localization was also usually well supplied by sinusoids outside the collection of primitive sympathetic elements. Thus, if released from the fetal adrenomedullary or paraganglionic CA-storing cells, the CA could easily reach the circulation. The cellular organization is very similar in this respect to the final circumstances in mature adrenal medulla.

CHAPTER II

ACTIVITY OF HISTOCHEMICALLY DEMONSTRABLE CHOLINESTERASES IN PARAGANGLIA AND ADRENAL MEDULLA

Introduction

Since it was demonstrated by several authors (for ref see Coupland 1965) that the adrenal medulla of all species studied possessed a sympathetic preganglionic innervation, the question of the nature of innervation of the paraganglia soon arose Hollinshead (1937 1940) assumed that at least the main para-aortic bodies possessed an innervation similar to that of the adrenal medulla Kofmann (1935) Pines (1924) Bakay (1937) and more recently Jacobowitz (1970) also reported contacts between sympathetic nerve fibres and the paraganglia

Brundin (1966) found indirect evidence of the absence of functional innervation of rabbit paraganglia. Again, the studies of Muscholl & Vogt (1964) gave no evidence for any functional innervation of paraganglia. Despite the light microscopical evidence nerve endings typical of preganglionic cholinergic innervation have not been found in rabbit (Coupland & Weakley 1968 1970) or mouse (Viragh & Korenyi Both 1965) paraganglionic tissue

In a preliminary report (Hervonen 1967) acetylcholinesterase (AChE) positive fibres were reported in human fetal paraganglia. Jacobowitz (1970) also found AChE positive fibres in abdominal paraganglia of cat

The release of CA from mature adrenal medulla is mainly regulated in physiological circumstances by the sympathetic innervation of the gland However very little is known about the reactions of human fetal CA-storing tissues to nervous stimuli. The purpose of the present investigation was to study the development of cholinergic innervation of fetal adrenal medulla and paraganglia by demonstrating both acetylcholinesterase (AChE) and nonspecific cholinesterase activity (nsChE)

Method

Both immersion and combined perfusion immersion fixation were used. The fixation was started within 10 min of the disconnection of fetoplacental circulation

The specimens were prepared after initial short perfusion with the fixative the adrenal glands cut in slices of 1 mm, and the paraganglia removed by preparing the internal preaortic region. The fixation solution was 3—3.5 % formaldehyde in 0 075 M phosphate buffer with 0.3 M sucrose or calcium formol (containing 1 vol of 35 % HCHO 6 vol of 2 % CaCl and 3 vol H O) The pH of buffered fixative was adjusted at 7 0 The fixation was performed at 4 C for 2—6 hours.

For the demonstration of AChE acetylthiocholine iodide (Fluka A G Buchs, Schweiz) was used as a substrate and 10^{-3}—10^{-4} iso-ompa (M tetra isopropylpyrophosphoramide L. Light and Co., Colnbrook, England) to inhibit nonspecific cholinesterase (nsChE) The nonspecific cholinesterase was demonstrated using butyrylthiocholine iodide (Fluka A G Buchs, Schweiz) as a substrate and 1 10^{-4}—10^{-5} 284C51 (M I 5-bis-4-allyl dimethylammoniumphenyl pentan 3-one Burroughs and Wellcome London) as a specific inhibitor for AChE

The Gomori (1952) modification of the Koelle (1951) technique was used. Incubation was performed at 37°C at pH 6 0—7 0 for 1—12 hours, using both free-floating and glass attached sections.

Results

Acetylcholinesterase

The primitive sympathetic trunk of an 8 weeks old fetus showed a clear though weak reaction (Fig 19) The network of AChE positive fibres extended throughout abdominal preaortic region The collections of primitive sympathetic cells could be observed as completely negative areas among the nerve plexus (Fig 19) A few fibres were found to invade the adrenal cortical elements from the medial side. The extra-adrenal primitive sympathetic cell groups were in tight contact with the primitive sympathetic plexus.

During the 9th week the invasion of AChE positive fibres towards the centre of adrenal glands was clearly observed Along their course through the cortical area very little branching of the positive fibres was observed, but an anastomosing network was found in the premedullary zone. Groups of primitive sympathetic cells could be found along the course of the fibres, and fine fibrous activity was also observed between the cells of these groups. During the 10th week more positive fibres were found inside the collections of primitive sympathetic cells and the intensity of this activity also increased (Fig 26)

Simultaneously during the 9—10th week, a clear organisational division of extra-adrenal primitive sympathetic elements into two parts occurred.

The "CA-storage-line" of differentiation of the cells developing into abdominal paraganglia consisted of large groups of completely AChE-negative cells. Nerve fibres were not found inside the groups, apart from solitary large trunks penetrating the bodies without any branching.

Groups of primitive sympathetic cells intensively invaded by AChE-positive fibres were seen probably representing the developing sympathetic ganglion. No cytoplasmic activity was observed in this group either. In general the intensity of the AChE activity both in the extra-adrenal sympathetic plexus and in the nerve fibres growing into the gland itself increased during the 8—10th week when it reached a level from which only minor variations were observed later. Instead the thickness of the nervous trunks and the tightness of the anastomosing networks both intra- and extra-adrenally increased greatly during the third month.

A large tight mesh of primitive sympathetic ganglionic elements was found in the inter-adrenal area. Before the 10th week of development no cytoplasmic activity was found within the primitive sympathetic ganglion. Several bundles of thick AChE positive fibres were found to reach the medullary anastomosing network in the adrenals of 12 weeks old fetus. At the age of 12 weeks the adrenal premedullary area assumed the following characteristics:

A tight network of anastomosing AChE positive fibres formed a clearly delimited, three-dimensionally tetrahedron-like primitive medulla (Fig. 25). Very few nerve fibres were found in the outer layers of fetal cortex apart from the medial aspects, through which the nerves reached the medulla (Fig. 28). The number of fine AChE positive fibres was increased and activity was always found also between the individual primitive sympathetic cells (Fig. 24 and 26). Finer ramification was also found between the groups of primitive sympathetic elements corresponding the distribution of CA-storing cells after the 10th week.

As a result of the condensation of the medulla the majority of primitive medullary elements were found in the central region. The fine AChE

Fig. 18. The [illegible] para-aortic body of 8 weeks old fetus. The nonspecific [illegible] reaction is positive on the capillary walls and supporting elements. 10 ×

Fig. 1[illegible]. The [illegible] para-aortic body of 8 weeks old fetus. The primitive sympathetic trunk showed moderate strong AChE positivity while the paraganglionic tissue was negative ([illegible] paraganglion.) 10 ×

Fig. 20. The [illegible] AChE activity in the paraganglia of 1[illegible] weeks old fetus. The positive reaction [illegible] in reticulum of supporting elements. 330 ×

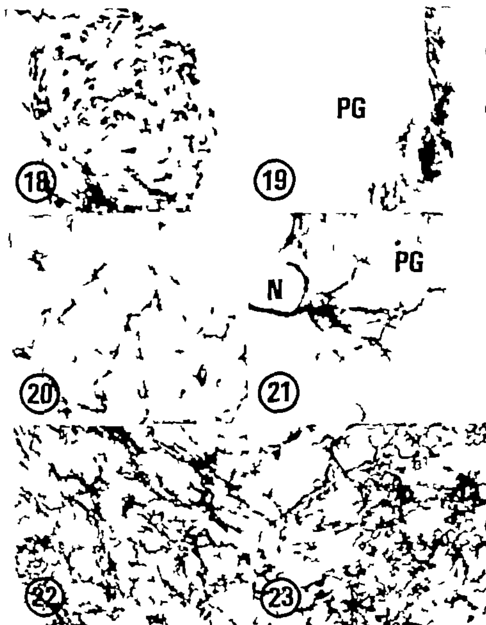


Fig 21. AchE positive fibres entering the main paraganglion from th sympathetic plexus. Note the extensive ramification f the fibre between the paraganglionic cells. (N=nerv fibre, PG=paraganglion.) 330 ×

Fig 22 Strong intercellular no ChE activity in the para-ganglion f 18 weeks old fetus. The reaction was positi in supporting elements and on the walls of capillary sinusoids. 220 ×

Fig. 23 Paraganglion f 18 weeks ld fetus. Cross sections of fibres which are AChE positive Positive reaction also long the capillary walls. 220 ×

positive branches were seen to be in contact with groups of CA-storing cells scattered through the premedullary area No cytoplasmic AChE activity could be demonstrated at the age of 1_ weeks in adrenal medulla

Instead, extra-adrenally a weak cytoplasmic activity of primitive sympathetic ganglionic cells was noticed at several levels of the sympathetic trunk. The ganglionic elements were in close contact with the para-aortic bodies, but no intermingling of these two cell-types was found The paraganglia always formed clearly restricted groups, with sharp boundaries towards the sympathetic ganglionic elements.

After the age of 1_ weeks, however solitary AChF positive fibres were found to extend from the surrounding sympathetic plexus to the outermost layers of the main para-aortic body (Fig. 21) These extra-adrenal nerve fibres could always be found during later stages, but their amount and the intensity of AChE activity did not increase markedly after the first appearance (Fig 23)

In spite of the rapid growth of paraganglia the tightness of the network of AChE positive fibres remained essentially the same The intensity of the reaction increased very little during the following three months. The fibres could be noticed at the 1?th week mainly within the outer layers of the paraganglia (Fig 21) but during the fourth month a gradual invasion of the entire tissue occurred. Occasionally thin branches from the coarse sympathetic fibres penetrating the bodies were found

The final degree of development of the AChE activity seemed to be reached rather early at the age of 16—18 weeks. The activity within paraganglia was still only in fibres, although the sympathetic ganglionic cells in the vicinity of paraganglia were positive The sparse network of fine and tiny fibres extended through the chromaffin tissue (Fig 23) The cross sections of fibres were frequently associated with the vascular walls, but no regularity in their course or organization could be demonstrated.

Th sympathetic elements of the preaortic area surrounding the paraganglia showed intense activity both cytoplasmic and fibrous (to be described in detail elsewhere)

After the 1?th week of pregnancy the condensation of adrenal medul

Fig. 21 A collection f primitiv sympathetic elements in drenal medulla of 1 week old fetus. Th A hE positive nerv fibres sent fine branches between the tightly packed primiti sympathetic cells. (PSC=primitive sympathetic lements.) 450 ×
Fig 22 Small groups of medullary CA-storing cells which give positive reaction f r AChE. Note the variable i tensity from cell to cell. 450 ×
Fig. 23 A small collection f primitiv sympathetic cells on medullary nerve fibre. AChE reaction. 14 weeks-old fetus (PSC=primitiv sympathetic cells.) 330 ×

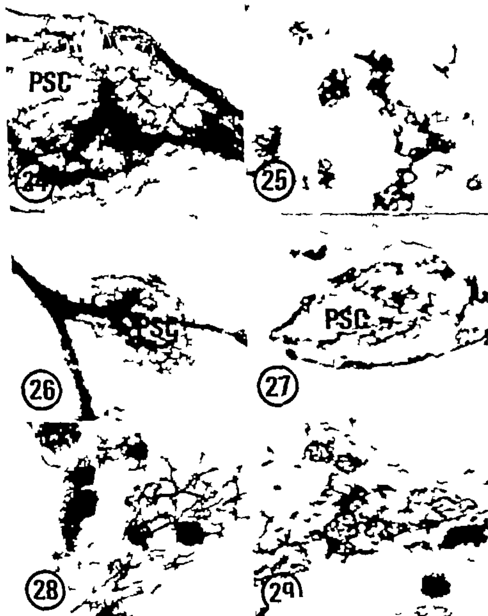


Fig. 27 N ChE activity in the dreonomedullary collection of primitive ympathetic cells. A rim of moderaty strong activity surrounds the group short streaks of activity also in th central region. (PSC = primitive sympathetic cells.) 120 ×

Fig. 28 The adrenal medulla of 16 week-old fetus. AChE reaction. Several large groups of primitiv ympathetic lements collected on the central area. The coarser fibres branch forming fine network both in the collections f primitiv cells nd on the medullary cells. 70 ×

Fig. 29 The same adrenal medulla, as. ChE activity. The reaction product forms *surrounding* rim around th primitive sympathetic elements. *Smaller* spots of activity on the sites of medullary cell groups. 70 ×

Discussion

The assumptions concerning the innervation of fetal CA-storing tissues have been studied by demonstrating the AChE and nsChE activities.

On the role of AChE and nsChE during development

Nerve fibres which contain acetylcholinesterase are very probably cholinergic (for ref. see Frankö 1967a). Thus the demonstration of AChE activity in tissues serves as a means to study the distribution of cholinergic nervous pathways. In adult tissues, AChE is selectively present in nervous tissues and nsChE dealing with the supportive nonneuronal structures. However, several roles for AChE during ontogenesis has been suggested by various authors (for ref. see Silver 1971). The metabolic and possibly other functions of AChE were not considered in this connection and the presence of the enzyme in certain localizations was assumed to indicate a possibly functioning cholinergic innervation. The function of nsChE in both adult and fetal tissues is very little understood (Silver 1971).

Innervation of the paraganglia

An AChE positive network of fibres was constantly found in para ganglia after the 1?th week of development, as stated before by the present author (Hervonen 1967). The activity in these fibres was weak and the network formed by them was sparse. The present results confirmed the findings of previous authors (Pines 19?4, Korfman 1935, Bavax 1937). Hollinshead (1937) supposed that the fibres were similar to those found in adrenal medulla. Jacobowitz (1970) demonstrated diffuse AChE-positivity in the abdominal paraganglia of the cat and suggested that these nerve fibres were preganglionic.

Muscholl & Vogt (1964) made stimulation experiments with chromaffin elements of cat and found no evidence for nervous regulation of catecholamine secretion. Brundin (1966) found no response to hypoglycemia, the effect of which is known to be mediated by nerves. More evidence of the nature of innervation of fetal chromaffin tissue has accumulated from the electron microscopic work of Coupland & Weakley (1968, 1970). They found axons but not cholinergic nerve endings in para-aortic bodies of the fetal rabbit. Similar results for human fetal extra-adrenal chromaffin tissue will be presented in Chapter IV.

Thus, the paraganglia of human fetus have an innervation which is probably cholinergic. However the functional role of this innervation is obscure The possibility of sensory function should not be neglected.

Innervation of fetal adrenal medulla

Although a differentiational delay in the development of FIF of adrenomedullary cells was clear the AChE activity around the medullary elements was stronger at all stages of development than that in the paraganglia. A tight network of anastomosing nerve fibres developed during the third month of development while the para-aortic bodies were devoid of innervation. The gross characteristics of the present AChE patterns were rather similar to those described by Eränkö *et al* (1959) Human fetal adrenal gland has not previously been studied with the present methods.

The fine branches of the main nervous network were found on the same areas as CA-storing cells. The amount of the fine branches increased gradually during the period studied, probably indicating the slow maturation of the medullary nervous supply The fine strands arising from the coarse medullary net were considered (Coupland 1965) as end branches on the medullary cells. The data presented in Chapter IV support this concept, suggesting that a contact between nerves and CA-storing cells might already be functional during the fourth month of development.

The AChE activity within the primitive sympathetic cell groups was rather similar to the circumstances in extra-adrenal sympathetic ganglion. The cells were gradually pulled apart from each other by AChE positive fibres, but no clear neuron figures were found intra-adrenally In the sympathetic ganglion, however primitive neurons showed positive reaction after the third month of pregnancy The tight contact of fine nerve fibres with the adrenal primitive sympathetic elements is further discussed in Chapter IV

nsChE activity

A weak positivity was noted in the cytoplasm of paraganglionic cells after the fourth month. Intra-adrenally small groups of medullary cells with moderate reaction could also be found regularly These findings are in agreement with those of Eränkö (1959) Palkama (1967) as well as Lewis and Shute (1969) who found nsChE activity in the endoplasmic

positive branches were seen to be in contact with groups of CA-storing cells scattered through the premedullary area. No cytoplasmic AChE activity could be demonstrated at the age of 12 weeks in adrenal medulla.

Instead, extra-adrenally a weak cytoplasmic activity of primitive sympathetic ganglionic cells was noticed at several levels of the sympathetic trunk. The ganglionic elements were in close contact with the para-aortic bodies, but no intermingling of these two cell types was found. The paraganglia always formed clearly restricted groups, with sharp boundaries towards the sympathetic ganglionic elements.

After the age of 12 weeks, however, solitary AChE positive fibres were found to extend from the surrounding sympathetic plexus to the outermost layers of the main para-aortic body (Fig. 21). These extra-adrenal nerve fibres could always be found during later stages, but their amount and the intensity of AChE activity did not increase markedly after the first appearance (Fig. 23).

In spite of the rapid growth of paraganglia the tightness of the network of AChE positive fibres remained essentially the same. The intensity of the reaction increased very little during the following three months. The fibres could be noticed at the 12th week mainly within the outer layers of the paraganglia (Fig. 21) but during the fourth month a gradual invasion of the entire tissue occurred. Occasionally thin branches from the coarse sympathetic fibres penetrating the bodies were found.

The final degree of development of the AChE activity seemed to be reached rather early at the age of 16—18 weeks. The activity within paraganglia was still only in fibres, although the sympathetic ganglionic cells in the vicinity of paraganglia were positive. The sparse network of fine and tiny fibres extended through the chromaffin tissue (Fig. 23). The cross sections of fibres were frequently associated with the vascular walls, but no regularity in their course or organisation could be demonstrated.

The sympathetic elements of the preaortic area surrounding the paraganglia showed intense activity both cytoplasmic and fibrous (to be described in detail elsewhere).

After the 1?th week of pregnancy the condensation of adrenal medul

Fig. 24. A collection of primitive sympathetic elements in adrenal medulla of 18 week old fetus. The AChE positive nerve fibres sent fine branches between the tightly packed primitive sympathetic cells. (PSO=primitive sympathetic elements.) 450 ×

Fig. 25. Small groups of medullary CA storing cells which give positive reaction for no ChE. Not the variable intensity from cell to cell. 400 ×

Fig. 26. A small collection of primitive sympathetic cells on a medullary nerve fibre. AChE reaction 14 weeks old fetus. (PSO=primitive sympathetic cells.) 330 ×

to those described for AChE. A few coarse trunks of nsChE activity already extended from the interadrenal primitive sympathetic plexus towards the centre of the cortical elements at the age of 8 weeks. The walls of capillary sinusoids of the medullary area were negative at all stages of development. At the age of 8—9 weeks, the groups of primitive sympathetic cells invading the cortex and collecting in the medullary area did not show activity.

The nsChE positivity in the paraganglia was still intercellular at the age of 12 weeks and probably restricted to the supporting elements of the para-aortic bodies (Fig. 20). No cytoplasmic activity was found. Instead the streaks of positivity were evenly scattered throughout the paraganglionic tissue. The intensity of the reaction increased slowly during the third month of development. In the developing sympathetic ganglion of a 12 weeks old fetus, cytoplasmic activity of moderate intensity was noticed in the ganglion cells between strongly positive fibres of the sympathetic trunk.

At the end of the 12th week, the distribution of intra-adrenal activity was the following. Coarse trunks of nerves were found to penetrate the cortex and reach the premedullary area (Fig. 29). The groups of primitive sympathetic cells were surrounded by a rim of positivity (Fig. 27). Streaks and diffuse patches of positivity were scattered through the premedullary area. Although no clear cytoplasmic activity was found, this patchy activity was evidently associated with the medullary CA-storing cells. The fine network of AChE positivity (Figs. 24 and 26) was not visible in the nsChE specimens.

In the paraganglia a clear intensification of the intercellular activity occurred after the third month of pregnancy. Weak cytoplasmic activity could be found after the 16th week (Fig. 22). Only the nuclei of paraganglionic chromaffin cells remained completely negative. The vascular walls were positive, as were the supporting elements extending between the paraganglionic cells. The cytoplasmic positive reaction remained very weak but was definitely specific.

Intra-adrenally the nsChE activity was found in three positions: (1) in the coarse nerve trunks penetrating the gland from the medial aspect of it; (2) the rim of activity surrounding the groups of primitive sympathetic cells (Figs. 28 and 29); (3) solitary streaks of activity corresponding to the distribution of medullary cells. In the oldest fetuses, clear cytoplasmic activity was demonstrated in at least some of the adrenomedullary CA-storing cells (Fig. 25). Cellular profiles were also found around the primitive sympathetic cell-groups.

Discussion

The assumptions concerning the innervation of fetal CA-storing tissues have been studied by demonstrating the AChE and nsChE activities.

On the role of AChE and nsChE during development

Nerve fibres which contain acetylcholinesterase are very probably cholinergic (for ref. see Eränkö 1967a) Thus the demonstration of AChE activity in tissues serves as a means to study the distribution of cholinergic nervous pathways. In adult tissues, AChE is selectively present in nervous tissues and nsChE dealing with the supportive nonneuronal structures. However several roles for AChE during ontogenesis has been suggested by various authors (for ref see Silver 1971) The metabolic and possibly other functions of AChE were not considered in this connection and the presence of the enzyme in certain localizations was assumed to indicate a possibly functioning cholinergic innervation. The function of nsChE in both adult and fetal tissues is very little understood (Silver 1971)

Innervation of the paraganglia

An AChE positive network of fibres was constantly found in paraganglia after the 12th week of development, as stated before by the present author (Hervonen 1967) The activity in these fibres was weak and the network formed by them was sparse. The present results confirmed the findings of previous authors (Pines 1924 Korfman 1935 Bavax 1937) Hollinshead (1937) supposed that the fibres were similar to those found in adrenal medulla. Jacobowitz (1970) demonstrated diffuse AChE positivity in the abdominal paraganglia of the cat and suggested that these nerve fibres were preganglionic.

Muscholl & Vogt (1964) made stimulation experiments with chromaffin elements of cat and found no evidence for nervous regulation of catecholamine secretion Brundin (1966) found no response to hypoglycemia the effect of which is known to be mediated by nerves. More evidence of the nature of innervation of fetal chromaffin tissue has accumulated from the electron microscopic work of Coupland & Weakley (1968, 1970) They found axons but not cholinergic nerve endings in para-aortic bodies of the fetal rabbit. Similar results for human fetal extra-adrenal chromaffin tissue will be presented in Chapter IV

Thus, the paraganglia of human fetus have an innervation which is probably cholinergic. However the functional role of this innervation is obscure The possibility of sensory function should not be neglected

Innervation of fetal adrenal medulla

Although a differentiational delay in the development of FIF of adrenomedullary cells was clear the AChE activity around the medullary elements was stronger at all stages of development than that in the para ganglia A tight network of anastomosing nerve fibres developed during the third month of development while the para-aortic bodies were devoid of innervation The gross characteristics of the present AChE patterns were rather similar to those described by Franko *et al* (1959) Human fetal adrenal gland has not previously been studied with the present methods.

The fine branches of the main nervous network were found on the same areas as CA-storing cells. The amount of the fine branches increased gradually during the period studied, probably indicating the slow maturation of the medullary nervous supply The fine strands arising from the coarse medullary net were considered (Coupland 1965) as end branches on the medullary cells. The data presented in Chapter IV support this concept, suggesting that a contact between nerves and CA-storing cells might already be functional during the fourth month of development.

The AChE activity within the primitive sympathetic cell groups was rather similar to the circumstances in extra-adrenal sympathetic ganglion The cells were gradually pulled apart from each other by AChE positive fibres, but no clear neuron figures were found intra-adrenally In the sympathetic ganglion, however primitive neurons showed positive reaction after the third month of pregnancy The tight contact of fine nerve fibres with the adrenal primitive sympathetic elements is further discussed in Chapter IV

nsChE activity

A weak positivity was noted in the cytoplasm of paraganglionic cells after the fourth month. Intra-adrenally small groups of medullary cells with moderate reaction could also be found regularly These findings are in agreement with those of Eränkö (1959) Palkama (1967) as well as Lewis and Shute (1969) who found nsChE activity in the endoplasmic

reticulum of rat adrenal medullary chromaffin cells. Coupland (1965) stated that chromaffin cells in general should not give positive reaction for nsChE if the reaction is performed in optimal conditions.

In the paraganglia the strongest reaction was given by the supporting elements and vascular walls. Intra-adrenally the reaction around the primitive sympathetic cell groups was presumably partly due to the surrounding connective tissue elements (see Chapter IV). The solitary streaks of activity were distributed in the areas where the fluorescent medullary cells could also be demonstrated, and were probably induced by activity in the Schwann elements (Lewis & Shute 1969, Palkama 1967). The vascular walls of the adrenomedullary sinusoids did not show any positivity as was the case in paraganglia. This difference in the activity of capillary walls might reflect some differing functional characteristic in these two organs. The general pattern of AChE/nsChE activities was basically similar to that found in rat adrenal medulla by Fränkö (1959). The coarse trunks showed both activities, but the fine fibres in the primitive sympathetic cell groups and around the groups of CA-storing cells showed only AChE positivity. This correlates well with the distribution of Schwann elements (see Chapter IV), suggesting that the nsChE activity might partly be due to these cells. The vascular reaction found by Coupland (1965) in the NA-storing regions was not observed.

The nsChE activity in the primitive sympathetic cell groups formed a strong surrounding rim around the vascular areas. This might reflect metabolic differences induced by changed differentiational patterns on the outer surface of the cell collection.

CHAPTER III

VASCULAR SUPPLY OF PARAGANGLIA AND ADRENAL MEDULLA

Introduction

The special features of adrenomedullary circulation have been studied by several investigators (for ref see Harrison 1960 and Coupland 196.) and the relations of adult chromaffin tissue to the circulation have been cleared up When the potential of the endocrine function of human fetal CA-storing cells is considered, the relations of both intra and extra adrenal CA-storing cells to the circulatory system are important. In the classic studies on the para-aortic bodies, Zuckerkandl (1901) and Kohn (1903) pointed out the rich vascular supply of the paraganglia. Recently Winckler (1969) studied the relations of extra-adrenal chromaffin cells to the capillaries in the rat and demonstrated an intimate contact between the wide capillary sinusoids and chromaffin cells. He suggested an effi cient release or uptake of products of metabolism after humoral stimula tion However no studies dealing specifically with the microvascular patterns of human fetal CA-storing tissue are available except the preliminary report of the present author (Hervonen & Suoranta 1971)

Lempinen (1964) found that the administration of cortical hormones to the rat caused the differentiation of new chromaffin cells from the primitive sympathetic elements or from some intermediate forms After the observation of Eränko, Lempinen & Räisänen (1966) and Coupland & McDougall (1966) that the cortical hormones exerted a direct regula tory effect on the formation of adrenaline from noradrenaline, the corticomedullary circulation was seen in a new light It was suggested by Wurtman and Axelrod (1965) that the local concentration of corticosteroids in adrenal medulla activated the enzyme phenylethanolamine N methyl transferase, and thus accelerated the methylation of NA to A. Thus, evidence has accumulated for the effect of humoral factors on the final maturation of CA-storing cells (for ref. see Roffi 1968) The steroidogenic activity of human fetal adrenal cortex probably starts as early as in the 7—8th week of gestation (Johannisson 1968 Saure & Pesonen 1969 Saure personal

communication 1971) Furthermore unbound steroids are able to cross the placental barrier to the fetal circulation, which allows the maternal hormones to act on the fetal tissues very early (Jost 1966) To summarize the possibility of humoral regulation of the earlier stages of differentiation of human fetal CA-storing cells as well (Hervonen 1971 a b) was taken into consideration and the relations of developing CA-storing cells to the microcirculation were considered particularly in terms of the cytodifferentiation

Methods

Microangiography

A modification of the method published by Kormano (1967a) was used. The angiographic procedures were started within 30 min of the disconnection of fetoplacental circulation. Experiments were also made with fetuses stored at 4 C at —20°C but capillary filling defects and extravasation were frequently observed in these fetuses.

A simple perfusion apparatus with adjustable pressure was constructed. 5—8 % $BaSO_4$ in 0.9 % NaCl was used as contrast medium (Kormano 1967b) A thin vinyl catheter was introduced into the umbilical artery or thoracic aorta of the fetus and the venous side of the circulation was opened. Perfusion time was 30—120 min and pressure 30—40 cm H_2O The specimens were fixed in situ by injecting 4 % buffered formalin solution into the abdominal cavity After half an hour the aorta was closed by knots to prevent leakage of $BaSO_4$ and the retroperitoneal tissue block containing adrenals, renals and main blood vessels was prepared. After further fixation of 2—4 days the specimens were embedded in paraffin.

For radiography sections 500 microns thick were cut and neighbouring sections 10 microns thick were stained with hematoxylineosin for light microscopy The 500 micron sections were placed in contact with a high resolution emulsion (Kodak, maximum resolution plate) A Siemens AG Cu 35 X ray tube with a copper target and a 0.5 mm beryllium window was used for microangiography The film focus distance was 100 cm and the exposure time 200 min at 35 kV and 10 mA. After developing the high resolution plates with Kodak D 19 developer they were photographed and studied in the usual way with light microscope.

Indian ink injection technique

A diluted special indian ink (Pelikan Spezialtusche C 11/1431) was injected into the umbilical artery of the foetuses. The injection time was from 1—5 min and the procedure was finished when the cutaneous capillaries observed under a preparational microscope were completely filled. The target organs were fixed in situ with 3.5 % buffered formalin. After —6 days a block containing the retroperitoneal tissues was embedded in paraffin The blocks were cut serially at 5—8 microns and stained with hematoxylin eosin. Special care was taken to prevent leaks of indian ink during the procedure The light microscopy and photography were performed in the usual way

Results

The paraganglia

Indian ink technique Because of the extreme fragility and extravasation tendency of the newly formed capillaries, the microangiographic technique could not be used for the youngest fetuses. A superior filling of the primitive capillaries was achieved with injections of indian ink.

Capillaries filled with indian ink were found in the middle of the preaortic primitive sympathetic elements of 8 weeks old fetus. In the same specimens nuclear changes were found in the cells around the blood vessels (Figs. 30—32) larger and paler nuclei were identified. In the 10 weeks old fetus several rounded or ovoid regions were found, where the capillary supply was well developed and the cytologic difference from the neighbouring avascular areas was evident (Fig 33) Small basophilic nuclei typical of primitive sympathetic cells were also found in the vascularized area, but the great majority of the nuclei were typical of developing CA storing cells. The size of these well vascularized clusters of developing CA-storing cells increased rapidly and a rich capillary supply was always present (Figs. 34—36) The primitive sympathetic elements around the para-aortic bodies were evidently differentiating towards sympathetic ganglia. Only solitary thin capillaries could be found within the boundaries of the ganglionic elements (Fig 34) At the age of 10 weeks the difference in the vascular patterns of paraganglia and developing sympathetic ganglia was striking The capillaries of the paraganglia assumed a sinusoidal form, with wide lumen and richly anastomosing course (Figs. 34—36) The

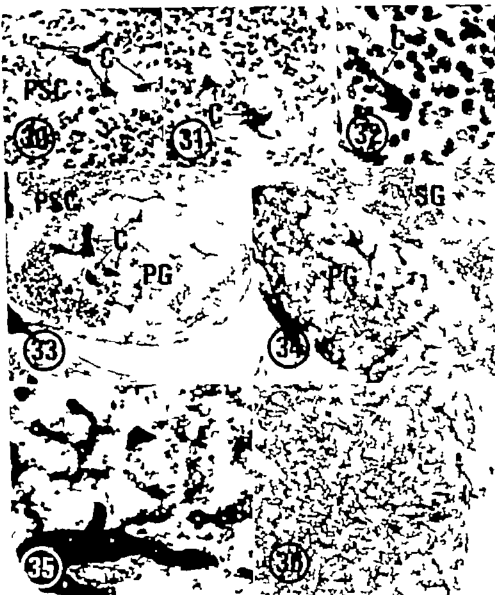


Fig 30 Capillaries filled with Indian ink i th middle f large preaortic mass of primitiv sympathetic tissue in a 8 weeks old fetus. Some larger and paler nuclei were found in the icinity f the essels. (C=capillaries) 230 ×

Fig 31 A capillary in the middle of th primitiv sympathetic tissue of 9 weeks-old fetus. Numerous larger nuclei were found near the capillaries. (C=capillary) 230 ×

Fig 32 Larger magnification from the same specimen as in fig 31 Nuclear changes visible (C=capillary) 460 ×

Fig 33 A rounded area of paraganglionic tissue in the middle of the primitive sympathetic region. Wide capillaries form rich network in the developing paraganglion. Fetal age 10 eeks. (C=capillaries, PG=paraganglion, PSC=primitive sympathetic tissue) 115 ×

Fig 34 A larger paraganglio f 1_ weeks-old-fetus A tight network of Indian ink

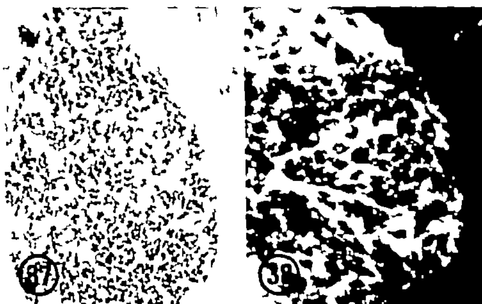


Fig 37 and 38 The paraganglion of a 14 weeks-old fetus. The microangiograph d the neighbouring section stained for light microscopy The widened capillaries re filled with the contrast medium in fig 37 The microangiograph show the corresponding anastomosing network f while sinusoidal capillaries. 170 ×

few capillaries found in the more primitive areas were thin and rarely branching

At the age of 1? weeks a rich vascular supply of CA-storing elements showed some regularities. Several arteriolar vessels were directed radially towards the centre of the paraganglionic cluster sending capillary branches along the course (Figs. 35 and 40) Larger arteries surrounded the bodies circularly The relative density of the capillary network increased evenly after the third month.

Microangiography After the age of 10—11 weeks the technical diffi culties caused by the extravasation of the contrast medium could mostly be avoided by careful regulation perfusion pressure and viscosity of the perfusate. The results obtained with indian ink injections could be con firmed (Fig. 39)

filled capillaries was regularly found. Different tion on the neuronal line was occuring i the vascular area surrounding th paraganglion. (PG=paraganglion, SG= ym pathetic ganglion.) 115 ×

Fig 38 The sinusoidal capillaries in the mal para-aortic body f 14 weeks ld fetus. A larger radial arteriole penetrates the paraganglionic of tissue giving short branches. 230 ×

Fig 39 The general view and the main para-aortic body of 16 weeks old fetus. An even uniform capillary network was found in all parts f the tissue. 120 ×



Fig. 30. Capillaries filled with Indian ink in the middle of large preaortic mass of primitiv sympathetic tissue in 8 weeks old fetus. Some larger nd paler nuclei were found in the vicinity of the vessels. (C=capillaries.) 230 ×

Fig. 31. A capillary in th middle of the primitive sympathetic tissue of 9 weeks-old-fetus. Numerous larger uclei were found near the capillaries. (C=capillary.) 230 ×

Fig. 32. Larger magnification from the same specimen as in fig. 31. Nuclear changes visible. (C=capillary.) 460 ×

Fig. 33. A rounded rea f paraga glionic tissue in the middle of th primitive sympathetic region. Wid capillaries f rm rich netwo k in the developing paraganglion. Fetal age 10 weeks. (C=capillaries, PG=paraganglion, PSC=primitiv sympathetic tissue.) 115 ×

Fig. 34. A larger paraganglion of 12 weeks-old fetus. A tight network f Indian ink

A clear difference between the vascular patterns of developing paraganglia and other primitive sympathetic elements was observed The paraganglia showed a very rich anastomosing sinusoidal capillary pattern at 1_ weeks (Fig 40) The circular main arteries gave out numerous radial branches which reached the central region of the organ The efficient branching of the radial arteries connected them with the capillary sinusoidal network. In the sympathetic ganglionic tissue a typical microvascular organisation was also observed The capillaries branched randomly from the main arteries, assuming a slowly coiling course The vessels were thin and the branching frequency was much less than in the paraganglia

The final characteristics of the vascular patterns of paraganglia were the following

The gross vasculature — The afferent arteries originating directly from the aorta run as two to three branches longitudinally along the main para aortic body Numerous circular branches arose along its course and they branched further thus forming a rather tight network of surrounding arteries (Figs. 40 and 42) The numerous radial arteries directed to the centre of the paraganglion were either short terminating in the superficial layers, or reached the centre of the body Thus, all layers of the paraganglia seemed to have equal vascular supply

The sinusoidal patterns — The radial arterioles sent out several short immediately anastomosing sinusoids. Th capillary net was similar throughout the para-aortic bodies (Figs. 37 and 38, 41 and 4) No changes were observed in this vascular organisation with increasing age of the fetuses, but the tightness of the sinusoidal network increased with age (Figs. 41 and 42)

The adrenal glands

The sinusoidal system of the fetal adrenal cortex was already well filled with the contrast medium at the age of 8 weeks. The development of cortical sinusoids is described in detail elsewhere. The main attention is now directed to the relations of medullary elements, the CA-storing cells and groups of primitive sympathetic cells to the circulation. The following main features were observed

The medullary CA-storing cells were found to be in intimate contact

---

Fig 41 A larger magnificatio of paraganglion of 14 weeks old fetus. The tight anastomosing capillary network clearly isible. 220 ×

Fig 42 The main paraganglia from a 18 weeks old fetus. The capillary network covers the whole paraganglia. Not the circular and radial branches f the afferent arteries. 100 ×

with the wide venous sinusoids of the premedullary area. The groups of primitive sympathetic elements formed completely avascular areas. The presence of capillaries inside the primitive sympathetic cell groups was particularly studied at all stages from the serially sectioned material. However even the largest collections of primitive sympathetic cells measuring about 1 mm in the oldest fetuses, were devoid of capillaries. In microangiographs the primitive sympathetic cell groups were seen as negative defects. The basic patterns of the medullary circulation, the arteries penetrating the cortex and ending directly at the medullary sinusoids, developed during the third month. The surrounding arteries sent out only short branches opening directly onto the cortical sinusoids before the age of 10 weeks, but during the third month longer thick branches (the arteriae medullae) were also found to reach the medullary area.

Discussion

Both well vascularized and poorly vascularized areas were found within the differentiating primitive sympathetic tissue in the para-aortic region. The developing paraganglia were supplied by fetal capillaries much more efficiently than the large mass of primitive sympathetic elements showing no signs of further differentiation. With increasing age the difference in the vascular supply persisted: the areas differentiating to the CA-storing cells (paraganglia) showed a well developed sinusoidal capillary system, while the differentiating sympathetic elements had no vascular supply at first, but a sparse network of thin primitive capillaries developed during the 9—12th weeks.

Comments on the vascular supply of fetal primitive sympathetic tissue were presented by Hervonen & Suoranta 1971. Several authors have found the rich vascular supply of para-aortic bodies (Kohn 1903, Zuckerkandl 1901, Iwanow 1934, Coupland 1952, Winckler 1969). Furthermore the "small intensively fluorescing cells" (SIF-cells) regularly found in the sympathetic ganglions of different species were in intimate contact with the capillaries (Eränkö & Härkönen 1963, 1965, Siegrist *et al.* 1968, Matthews & Rauman 1969, Jacobowitz 1970, Eränkö & Eränkö 1971). As regards the human fetal CA-storing tissue this statement may well be extended in the light of the present work to read that wherever collections of non-neuronal CA-storing cells (SIF-cells, chromaffin cells) were found in the para-aortic region or adrenal glands, a close contact with the capillaries could always be demonstrated.

The proximity of CA-storing cells to blood circulation provides suitable conditions both for release of products from the cells and for humoral stimuli to reach the cells. Both possibilities should be considered (see Chapter V)

Lempinen (1964) exhaustively discussed in the light of available data the possibilities of adrenocortical steroids, especially hydrocortisone, inducing the differentiation of new chromaffin cells from the primitive sympathetic elements. He found direct evidence for differentiation of new chromaffin cells in rat extra-adrenal chromaffin tissue and sympathetic ganglia after injections of hydrocortisone Erankö Lempinen & Räisä nen (1960) showed the regulating effect of hydrocortisone on the synthesis of adrenaline from noradrenaline in the rat paraganglia. The results were confirmed by Coupland *et al* (1966) with chromaffin cells cultivated *in vitro* Later several authors (for ref. see Roffi 1968) showed that the activity of the methylating enzyme phenylethanolamine-N-methyl transferase was dependent on the concentrations of hydrocortisone The present results showed that the development of CA-storing cells was at all stages if not preceded, at least accompanied by development of a rich capillary network. The presence of capillary supply evidently stimulated the metabolism of the well vascularized part of the developing primitive sympathetic elements and promoted the differentiation of CA-storing cells. The effect might hence be due solely to the oxygenation difference. However the previous data on the effect of glucocorticoids on the differentiating tissues lead to the conclusion that here too these hormones may be of decisive importance when the direction of differentiation of primitive sympathetic elements is determined.

The endocrine function of paraganglia was first suggested by the pioneers of this subject (Zuckerkandl 1901 Kohn 1903) and was indirectly supported by experimental data presented by many other investigators (for ref. see Coupland 1965) The present results, combined with the data from other chapters, show suitable conditions for release of catecholammes or other products (Hervonen 1971a) from the human fetal CA-storing cells.

CHAPTER IV

FINE STRUCTURE OF PARAGANGLIA AND ADRENAL MEDULLA

Introduction

The main characteristics of rat adrenomedullary cell have been described by several investigators (Eränkö 1960 Coupland 1965a b Elfvin 1965 Lewis & Shute 1969 Palkama 1967) The first investigation dealing specifically with the differentiational features was presented by Elfvin (1967) who followed the development of secretory granules in the cytoplasm of rat adrenal medullary cell. The first comment on the fine structure of rabbit paraganglia and adrenal medulla was made by Brundin (1965 1966) and the presence of typical "chromaffin cells in paraganglia as well was demonstrated. More recently Coupland & Weakley (1968 1970) studied the development of "chromaffin tissue of rabbit in these two localizations, and found that the main stages of differentiation observed by light microscopy could also be identified by EM Virágh & Korenyi Both (1967) studied the degenerational features in the paraganglia of postnatal mouse.

The emphasis in studies of extra-adrenal CA-storing cells has recently been on the small intensively fluorescing cells" (SIF) first described by Eränkö & Härkönen (1963) The SIF-cells were electron microscopically found to be largely identical with typical chromaffin cells of the adrenal medulla (Eränkö & Härkonen 1965) and the presence of catecholamine storing granules has since been confirmed by several authors in these cells (Grillo 1966 Williams 1967 Elfvin 1968 Siegrist *et al* 1968 Hökfelt 1969 Matthews & Raisman 1969 Williams & Palay 1969) The similarity of SIF cells, which often do not exhibit a positive chromaffin reaction, to the truly chromaffin cells is still a matter of discussion (Eränkö & Eränkö 1971)

The paraganglia which form the main part of extra-adrenal chromaffin tissue have inspired only a few studies other than those previously mentioned possibly because of the restricted period of persistence of the tissue. However for elucidation of the nature of SIF-cells and extra

adrenal CA-storing elements the developmental series of events is of importance

The only report on human fetal paraganglia was given by Battaglia (1969) who found mainly NA-storing granules in the paraganglionic cells. In a preliminary report (Hervonen 1970a) the early appearance of catecholamine storing granules in human fetal CA-storing cells was pointed out and the main characteristics of the innervation and ultrastructural differentiation have been characterized (Hervonen 1971a)

The purpose of the electron microscopic part of the present work was to check the results obtained with the light microscopic methods and answer the issues left open.

Methods

The processing of the material was started immediately after disconnection of feto-placental circulation (within 1—2 min of the first disturbance of fetal circulation during the operation)

Both immersion and perfusion fixation were used The small size and immaturity of the circulatory system of the smallest fetuses (see Table I fet. 1 2, 3, 5 and 6) meant that complete perfusion could not be performed Instead the retroperitoneal block containing the adrenals and the preaortic region was prepared the adrenal elements of one side and a piece of the preaortic region were processed for other purposes (see Table No 1) and the rest were immersed in the fixative. When perfusion fixation was used later the specimens for other methods were also rapidly prepared before the perfusion started.

A thin vinyl catheter or injection needle was placed either in the umbilical artery or the thoracic aorta. The venous side was opened by catheterizing the umbilical vein or opening the right atrium of the heart. Any leakages caused by the removal of adrenal and preaortic tissue for other purposes were closed The perfusion pressure was adjusted to between 30—100 cm H_2O depending on the age of the fetuses, and the perfusion time was 10—15 min For further immersion in the same fixative, slices 1 mm thick were cut sagittally from the medial and central parts of the adrenals, and the medullary region was prepared under stereomicroscope, under which the para-aortic bodies were also easily identified. The paired main mass of the main para-aortic body was removed and cut in pieces of about 1 mm The total fixation time with the primary solution was 2—4 hours.

The main fixation fluid was 2—2.5 % charcoal washed glutaraldehyde in 0.075—0.1 M phosphate buffered 0.3 M sucrose. For detailed studies on CA-storing vesicles 2 % potassium permanganate in 0.1 M phosphate buffer (Hökfelt 1960) a modification of Richardson's (1964) fixative was used. With glutaraldehyde as primary fixative postfixation with 1 % buffered OsO was used.

After the short dehydration procedure (Kokko & Reehardt 1968) the pieces were embedded in epon Epoxy resin (Luft 1961) or Epon Araldite mixture and sectioned with LKB or Reichert ultrotome. Sections for light microscopy were cut with LKB pyramitome. Both uranyl acetate and lead citrate or lead citrate alone (Reynolds 1963) were used for poststaining. The sections were studied and photographed with Philips EM 200 and 300 operated at different voltages (for EM 300 40—60 kV).

Results

General comments

The differentiation of a primitive sympathetic cell to a mature CA storing cell (chromaffin cell or SIF-cell) was regarded as a series of ultrastructural changes with preceding alterations in the chemodifferentiational patterns. When the para-aortic bodies or adrenal medullary tissue of a fetus representing a certain stage of development were studied several intermediate forms between the primitive sympathetic cell and mature CA storing cell were always found at the same time. The moment of onset of further differentiation of primitive sympathetic cells in different localizations seemed to vary considerably. The rate of differentiational events also probably differed from cell to cell. In the present study therefore, estimation of the order of appearance of new ultrastructural characteristics during the differentitation of the CA-storing cells was preferred to description of the different intermediate cell types.

Primitive sympathetic cells

The collections of primitive sympathetic cells were easily identified among the reticular mesodermal elements of the preaortic area (Fig 43)

Fig 43. Primitive sympathetic cells from the para-aortic region of 6 weeks old fetus. The cells were tightly packed mostly polygonal in shape. 7870 ×
Fig 44. A primitive sympathetic cell from the paraganglia of 7 weeks old fetus. A well developed Golgi apparatus with both lamellar and vesicular components visible. Note the microtubuli besides the Golgi apparatus. 14250 ×



Fig 45 A longitudinal section through the primitive sympathetic plexus passing by the paraganglia. The axons contained only bundles of neurofilaments at this stage of development. 32000 ×

The primitive sympathetic cells were present along the whole length of the abdominal aorta, but even in the youngest fetuses (Table I fet 1, 2, 3) larger groups were found at the level of the coeliac artery The cells were loosely packed with wide intercellular spaces. No desmosomal attachments were seen. The cells were usually elongated, and the ovoid nuclei were surrounded by only a thin rim of cytoplasm (Fig 43) The outlines of the plasma-membrane were irregular and cytoplasmic invaginations and extensions were frequently found. Myelin figures were observed in the intercellular spaces. Primitive axonal processes were also found to pass by the primitive sympathetic cell collections.

The cytoplasm was rather poor in organelles, but all the common ones were represented In the youngest fetuses a few ribosomes were found scattered evenly through the cytoplasm, but in the 7—8 week old ones the amount of both free and membrane-bound ribosomes was increased, as was the relative volume of the cytoplasm A well developed Golgi apparatus was found in the majority of the primitive sympathetic cells at 7 weeks (Fig 44) A smooth-surfaced membrane system was frequently found earlier but the typical Golgi apparatus with vesicular component was absent. Rough surfaced membranes were also found, and their relative amount seemed to increase slowly during the second month. Glycogen was frequently met after the 7 weeks stage usually in the form of alpha particles grouped tightly together at some corner of the cytoplasm This organization of glycogen was not found in the more mature cell, where the glycogen granules were more evenly scattered through certain cytoplasmic areas. A few mitochondria were present and occasional microtubuli could be found (Fig 44) The presence of a few microtubuli was a constant feature throughout the differentiation.

Primitive nerve bundles were found in close contact with the primitive sympathetic cell collections. The cross sections of the axons were usually empty showing only a few neurofilaments. The processes were packed in loose bundles and primitive Schwann-elements were found among the axons (Fig 45)

Distribution of primitive sympathetic cells

In the 7.3 and 8 weeks old fetuses the distribution of primitive sympathetic cells was specially studied by sectioning the specimens serially Groups of primitive sympathetic cells could be found along the nerve fibres deep in the adrenal cortical elements. Small electron dense cells, probably primitive sympathetic cells, were also found among the cortical

elements of the 6 week old fetus. The real invasion period seemed to be at the age of 5—8 weeks, during which time the amount of intracortical primitive sympathetic elements greatly increased and the cell groups also appeared in the centre of the gland.

Extra-adrenally the diffuse grouping of primitive sympathetic cells became more consolidated and clear boundaries to the surrounding connective tissue elements were found. Intimate contact with axon bundles was regularly noted.

Changes in the fine structure of primitive sympathetic cells

The essential features of the cytoplasmic patterns remained unchanged at both extra- and intra-adrenal localisations up to the 8th week of development. The nuclei of numerous cells showed clear increase in size and the density of the chromatin was decreased. The total cellular size was also slightly increased; there may have been slight changes of the cytoplasmic constituents. Possible examples of this were the increase in the density of ribosomes and amounts of vesicular component of smooth endoplasmic reticulum.

In the 8 week old fetus (No 6) a clear increase of smooth endoplasmic reticulum and ribosomes was noted. In particular the empty vesicles probably originating from the Golgi apparatus became dominant. The Golgi areas were regularly found and the proportion of vesicular component was high. The diameters of these empty vesicles were from c. 600—1400 Å. The vesicles were mostly round or slightly ovoid, but elongated and irregular forms were also occasionally found. In the 8 weeks old fetus, some occasional vesicles, partially filled with a dense material were found (Fig 46) in extra-adrenal primitive sympathetic cells. However the filling of the vesicles was not a regular observation before the 9 week old fetus. All degrees of filling of the vesicles with electron dense material could be found (Fig 48). The dense core might be small and rather dense, or might fill the vesicle almost completely showing variable degrees of density. Empty vesicles were also found. The dense-cored vesicles were not found in all individual cells studied. No other clear differences between the cells with and without dense-cored vesicles could be demonstrated.

In the 10 weeks old fetuses further differences between individual cells could be demonstrated. The nuclear size and total volume of the cytoplasm seemed to be increased in the majority of the cells. The CA-granules were also regularly found in the cytoplasm of the larger variety of cells, which



Fig. 46 Section through an early CA storing cells in the middle of more primitive sympathetic element in the preaortic paraganglia of 8 weeks old fetus. Electron dense material has accumulated in the vesicles formed by the smooth surfaced endoplasmic reticulum. (Arrow = CA granules.) 19300 ×

Fig. 47 A paraganglionic cell from 9 weeks old fetus. The CA-granules were found llower th cytoplasm. The electron density of the granular content varied greatly 19600 ×

Fig. 48 Section through a densely packed group of paraganglional cells of 9 weeks old fetus. The amount of CA-storing granules has increased greatly as also the amounts f ribosomes which frequently formed polysomal aggregates. The majority of the vesicular cores were rather electron dense and only few empty vesicles were found in the *cytoplasm.* 42300 ×

could now be termed granule-containing cells. The amount of primitive sympathetic cells in the main para-aortic body was decreasing rapidly.

Parallel to the increase in the amounts of CA-granules and the total volume of the cytoplasm, the relative amounts of ribosomes, rough endoplasmic reticulum and mitochondria also increased (Fig. 47). Ribosomes were scattered evenly through the cytoplasm and often formed polysomal aggregates. The rough endoplasmic reticulum was observed as solitary short streaks, but after the age of 12 weeks large areas of parallel lamellae became usual. The mitochondria were round or elongated in profile, showing no unusual features. Cytoplasmic aggregates of glycogen were frequently found, usually in the peripheral regions of the cytoplasm. The golgi apparatus formed a prominent feature consisting of vesicular and lamellar components as before. The number of empty vesicles scattered through the cytoplasm was decreased but the vesicular component of the golgi area was still well developed.

The shape of the nuclei showed no regular characteristics: they were usually elongated or ovoid, but round and extremely elongated figures were also found. The nuclear membrane assumed a more irregular course than in the primitive sympathetic cells, and invaginations of cytoplasm were frequent. The overall electron density of the nuclei of granule containing cells was less than that of primitive sympathetic cells. Instead of the former uniform ground darkness found in the nuclei of primitive sympathetic cells, the chromatin reticulum was more clearly visible. At the age of 12 weeks the chromatin was found to form a peripheral rim on the inner surface of the nuclear membrane. Usually 1—2 nucleoli were found.

The size of CA-granules increased during the differentiation (See table II). At the age of 9 weeks the mean diameter of the first dense-core vesicles was 1095Å, and in the early chromaffin cells of 12 weeks old fetus 1603Å.

Table II

Size of CA-granules in developing CA-storing cells

Age (weeks)	Paraganglion mean	σ	ADH medulla mean	σ
8—9	1095 Å	345 Å	—	—
10—11	1603 Å	630 Å	1528 Å	449 Å
12—13	2176 Å	656 Å	2090 Å	670 Å
14—15	2633 Å	828 Å	2506 Å	773 Å
16—17	2710 Å	850 Å	2825 Å	890 Å
18—19	2590 Å	837 Å	2664 Å	740 Å

(σ = standard deviation)

The fine structure of developing granule-containing cells was similar both in the paraganglia and within the cortical elements. However the differentiational patterns differed in some respects. The appearance of CA granules in the cytoplasm was delayed when compared with extra-adrenal development. At the age of 10 weeks when granulecontaining cells were regularly found in paraganglia only a few cells of this type were observed in the medial aspects of fetal cortex. Instead numerous large groups of primitive sympathetic cells were also found in the central region of the gland. Between the 10th and 12th weeks, however the number of granule containing cells greatly increased intra-adrenally as well. Despite the rapid differentiation of medullary elements, large groups of primitive sympathetic cells persisted intra-adrenally while they gradually disappeared in developing paraganglia.

Differentiation of intra-adrenal primitive sympathetic cells

The granule-containing cells found in the premedullary area of adrenal glands of 10 weeks old fetus were usually gathered in small groups along the venous sinusoids. Primitive sympathetic cells could be observed in close contact with the invading nerve fibres, but nerves could only occasionally be demonstrated in connection with the granule-containing cells. Cells both with and without CA-granules were also found in the same cell group. However a general division into two separate differentiational patterns was evident smaller groups of granule-containing cells and larger groups of primitive sympathetic cells showing no further signs of differentation at the age of 10 weeks.

The organization of primitive sympathetic cell groups — The intra adrenal primitive sympathetic cells were similar to the extra-adrenal ones. The cells, however were very tightly packed and only tiny intercellular spaces were found. Thus the cells and nuclei were usually round or polygonal in shape and very few elongated forms were found on the outer margins of the groups. Axon bundles frequently crossed the primitive sympathetic cell groups, giving solitary fibres between the cells. At the age of 9 weeks, however the branching of the cross trunks was not extensive and only a few solitary fibres were found in the intercellular spaces. During the 10th and 11th weeks, the amounts of axonal processes, as also the number of local cytoplasmic extensions (Fig 49) greatly increased, separating the individual cells (Fig 50) A rosette organization of primitive sympathetic cells was frequently found around the larger nerve fibres passing through the primitive sympathetic cell groups.



Fig. 49 Section through intra-adrenal collection of primitive sympathetic elements f 10 weeks ld fetus. The cells were tightly pa ked and polygonal. Not the cytoplasmic extensions. 29000 ×

Connective tissue elements, fibroblasts and collagen fibres were regularly found around the primitive sympathetic cell groups and larger nerve trunks. Schwann cells were identified after the age of 10 weeks on the axon bundles among the fetal cortex The groups of primitive sym pathetic cells were separated from the surrounding cortical cells of venous sinuses by a thin and irregular layer of collagen or fibroblasts. The con nective tissue capsule however was not continuous, and direct surface contacts between the cortical cells and primitive sympathetic cells were also regularly seen. The sympathetic elements were usually separated from the cortical ones in the 9—10-weeks old fetuses by a rather large intercellular space.

The primitive sympathetic cell groups were usually rounded and ovoid in shape, but extensively elongated forms were also found on the nerve trunks. Occasionally irregular groupings with both granule-containing

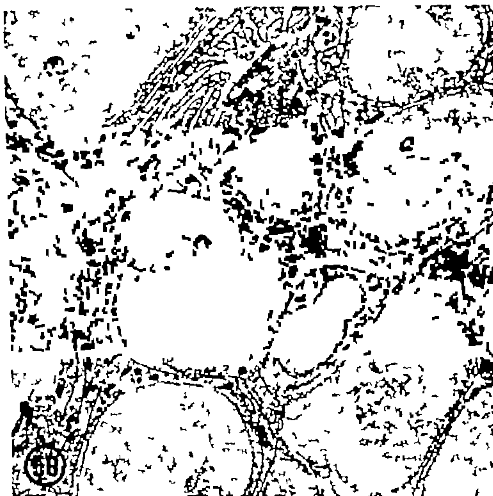


Fig 60 Section through a large adrenomedullary collection of primitive sympathetic cells. The cells were separated from each other by bundles of cytoplasmic processes. Bundles of preganglionic axons were found between the processes from primitive sympathetic elements 7800 ×

cells and primitive sympathetic cells were met The granule-containing part of these groupings had a direct surface contact with the primitive sympathetic cells, but no complete mixing of the two cell types, as found during the early stages in paraganglionic tissue was observed. Thus the primary patterns of differentiation of medullary elements seemed to be more separate from the primitive sympathetic elements in adrenal medulla

Differentiation into CA-storing medullary cells — After the 10 weeks stage of development it became evident that continuous differentiation occurred in the primitive sympathetic cell groups of adrenal medulla The

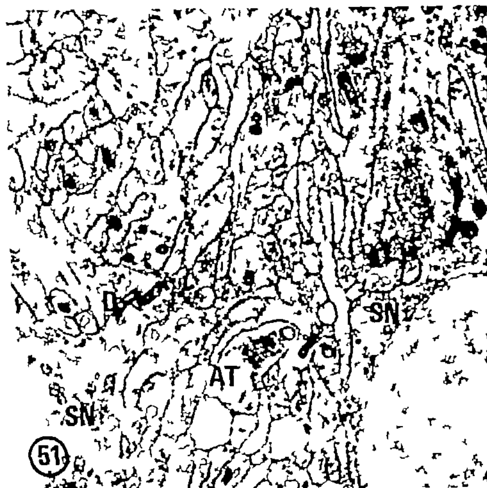


Fig. 51. A wide intercellular space from adrenomedullary collection of primitive sympathetic cells of 1[illegible] week old fetus. Cytoplasmic extensions contain microtubuli and small mitochondriae. A single free axon terminal with agranular synaptic vesicles and few dense cored vesicles was found between the processes. (AT=axon terminal, SN=Sympathetic neuroblast, D=Dendritic process) 16400 ×

large majority of the cells retained their primitive nature, but during the first half of the third month of development numerous cytoplasmic extensions developed from a proportion of cells mainly in the central part of the groups (Fig. 50). On the boundaries of the groups in 1[illegible] weeks old fetuses typical changes towards the granule containing cell were identified in solitary cells: appearance of CA-granules, increase of cellular size, increase of the cytoplasmic volume and amounts of organelles. Very soon at the age of 1[illegible] weeks, mature medullary granule-containing cells were found in the same localization inside the surrounding connective tissue capsule. Thus,

at least on the peripheral zone of the primitive sympathetic cell groups, differentiation towards medullary CA-storing cells occurred.

On the other hand very different changes were observed in numerous cells in the central region of the groups. Because of the characteristics assumed by these cells, they were named sympathetic neuroblasts.

Neuronal differentiation in adrenal medulla Parallel with the cytoplasmic ramification which started after the 10th week of development the total size of these cells slowly increased. The cytoplasmic processes could extend several microns between the adjacent cells and preganglionic axons (Fig 50) The processes were rather thick and contained the usual cytoplasmic organelles (Fig 51) A complex network of cellular processes and nerve fibres was formed widening the intercellular areas at some regions of the group (Fig 50 and 51) And bundles of microtubuli were found in the cellular processes (Fig 51) The preganglionic nerves could be identified by their lack of cytoplasmic constituents other than microfilaments or microtubuli and occasional CA-granules.

The final characteristics of a sympathetic neuroblast within the large collections of primitive sympathetic elements was not much different from the developing neurons in abdominal sympathetic ganglia

The nuclei of the neuronal cells were large and ovoid or round (Fig 52) The electron density of the nuclear material was decreased compared to the more primitive neighbouring sympathetic elements 2—4 nucleoli were regularly encountered, usually located on the inner surface of the nuclear membrane The chromatin reticulum was even and only a few coarser collections of chromatin were found in addition to the nucleoli

The overall shape of the cells was very varied The tightly packed organization of the sympathetic elements caused mostly a polygonal profile of the cellular soma. The cytoplasmic processes were both short and thick, with all common cytoplasmic organelles, or long and thin containing only microtubuli and a few ribosomes and mitochondria

The cytoplasm was much like the neuronal cytoplasm described by (Pick *et al* 1964) The rough endoplasmic reticulum was abundant and consisted of short streaks of double membrane scattered evenly throughout the whole cellular soma. Large amounts of free ribosomes were present, mostly forming polysomal aggregates solitary free ribosomes were also found. A golgi apparatus was regularly found, but was not so dominant as in developing CA-storing cells. The lamellar and vesicular components were both present

The mitochondria were small, with an electron dense matrix. The mitochondria were evenly distributed through the cellular soma. A few



Fig 52 Sympathetic neuroblasts in the central region of a large collection of primitive sympathetic cells in adrenal medulla of 16 weeks old fetus. Typical cytoplasmic patterns of developing sympathetic neuron were visible. The cells were surrounded by dendritic processes from neighbouring cells and by preganglionic axons. Note the synapse on sympathetic neuroblast. (SN = Sympathetic neuroblast, SY = Synapse.) 17300 ×

lysosomal particles were found. CA-storing dense cored vesicles were regularly found in the developing sympathetic neurons. The size of the vesicles was c. 980 Å. The amount of CA-storing vesicles in relation to cellular size remained unchanged, and their size did not increase with age

Bundles of microtubuli were observed in the cellular processes and

also in the cellular soma. Neurofilaments were also found in the older specimens.

Synapses were occasionally observed on the surface of sympathetic neuroblasts after the age of 14 weeks. These synapses were probably formed by a preganglionic axon (Fig 52 and 53)

Mature granule-containing cell

The majority of the adrenomedullary cells and paraganglionic cells are probably true chromaffin cells.

After the 12th—15th weeks the great majority of granule-containing cells in both paraganglia and adrenal medulla had assumed certain stabile characteristics and the cells were supposed to be morphologically mature (Fig 54—55)

When the overall appearance of the main para-aortic bodies was investigated, slight differences in the background darkness of individual cells could be observed. When non poststained sections were used the difference was lessened. Intra-adrenally where the cellgrouping was different, only the lighter variety of granule-containing cells was found.

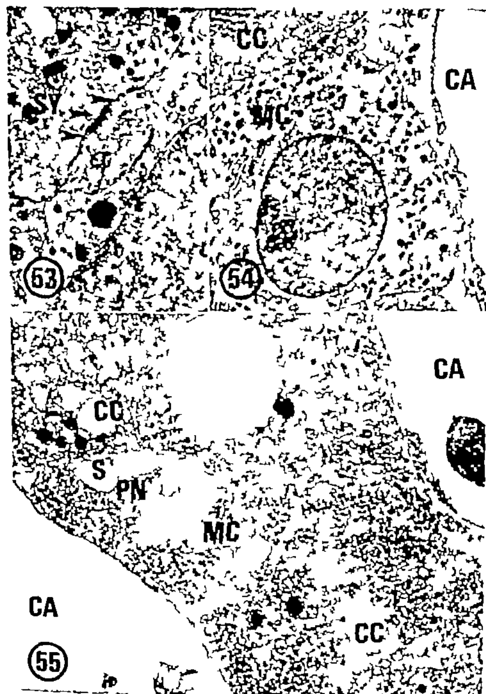
The general shape of mature granule-containing cells was widely dependent on the surrounding tissue. When located in the middle of larger collections the cells were rounded or polygonal owing to the tightly packed organization. Elongated and columnar forms were found on the venous sinusoids. The free margins of adrenomedullary cells towards the capillaries or adrenocortical cells were mostly smooth, but short cytoplasmic extensions could occasionally be found. Thin layers of collagen fibres and, rarely fibroblasts surrounded the separate adrenomedullary cell groups.

The relation of granule-containing cells to the local capillaries was similar in both paraganglia and adrenal medulla. A narrow intercellular gap usually separated the plasma membrane of CA-storing cells from the thin basement membrane of the capillary wall (Figs. 54 and 55) Connective tissue elements were found in this space However direct contacts of

Fig 53 The same synapse as in fig 52. Only agranular synaptic vesicles were found in this axon terminal (SY arrows=synaptic thickening) 31400 ×

Fig 54 A mature adrenomedullary CA-storing cell of 13 weeks old fetus. Note the close contact of the CA storing cell to the medullary capillary and to the fetal cortical cells. The cytoplasm was densely packed with CA-storing granules of NA type (CC= cortical cell, CA=capillary MC=medullary cell.) 6800 ×

Fig 55 Group of columnar adrenomedullary cell of 14 weeks old fetus. The cells possessed vascular contacts with two venous sinusoids. A cross section of a pregang-



lionic nerve bundle with surrounding schwann elements visible. The capillary w ll was rely fenestrated (CC=cortical cell, CA=capillary MC=medullary cells, PN=preganglionic nerv S=Cell f Schwann.) 3800 ×



Fig. 58. CA-storing cell inside the connective tissue capsel surrounding large collection of primitive sympathetic cells in adrenal medulla of 14 weeks old fetus. Thin layer of collagen fibres and fibroblasts separate the CA-storing cell from the cells of fetal cortex. (CC=cortical cell, MC=medullary cell, F=fibroblast, N=nervous processes.) 9840 ×

CA-storing cells and capillary basement membrane were also frequently found. In the intra-adrenal capillary endothelial cells, more pinocytotic vesicles were found in the regions where CA-storing cells faced the vascular wall.

The nuclei of CA-storing cells were round or ovoid and their diameter was double that of primitive sympathetic cells (Figs. 54, 55, 56). The shape of the nuclei was more regular than in the less mature cells, where cytoplasmic invaginations were more frequently found. The chromatin had condensed on the inner surface of the nuclear membrane. The central areas of the nuclei were lighter and chromatin formed small reticular aggregates.

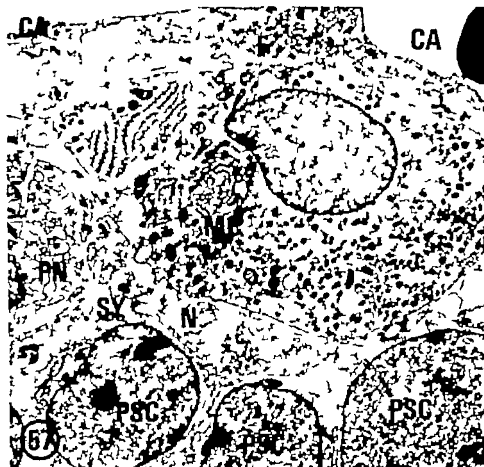


Fig 57 A typical intracapsular CA-storing cell in adrenal medulla of 16 weeks old fetus. Outside the connective tissue element two capillaries in close contact to the capsel. Note the primitive sympathetic cells, synaptic contact on the CA-storing cell and the cross section of bundle of preganglionic axons surrounded by cell of Schwann. (PSC=primitive sympathetic cell, MC=medullary cell, SY=synapse on the medullary cell, PN=preganglionic nerve CA=capillary F=fibroblast N=neuronal processes.) 8400 ×

The chromatin was dispersed more evenly through the nucleoplasm than in the immature forms and only a few larger aggregates were found. Usually one or two nucleoli were found, mostly peripherally or on the nuclear membrane. The nucleoli consisted of a reticular mass of relatively high electron density with pores of lower density (Fig 54) The overall electron density of the nucleoli increased with the maturation process.

The large CA-granules were the prominent features of the cytoplasm. They were evenly dispersed through the cytoplasm, avoiding only the Golgi area and the areas of tightly packed ergastoplasmic reticulum. Very



Fig. 56. CA-storing cell inside the connective tissue capsel surrounding a large collection of primitive sympathetic cells in adrenal medulla of 14 weeks old fetus. This layer of collagen fibres and fibroblasts separate the CA-storing cell from the cells of fetal cortex. (CC=cortical cell, MC=medullary cell, F=fibroblast, N=nervous processes.) 6840 ×

CA-storing cells and capillary basement membrane were also frequently found. In the intra-adrenal capillary endothelial cells, more pinocytotic vesicles were found in the regions where CA-storing cells faced the vascular wall.

The nuclei of CA-storing cells were round or ovoid, and their diameter was double that of primitive sympathetic cells (Figs. 54, 55, 56). The shape of the nuclei was more regular than in the less mature cells, where cytoplasmic invaginations were more frequently found. The chromatin had condensed on the inner surface of the nuclear membrane. The central areas of the nuclei were lighter and chromatin formed small reticular aggregates.



Fig 59 Paraganglionic CA-storing cell of 10 weeks old fetus. The cytoplasm was filled with CA-storing granules and glycogen. 4470 ×

inside the Golgi membrane systems, but usually they were empty The rough surfaced ER was also well developed consisting of parallel lamellae. Shorter streaks scattered through the cytoplasm were also found. Like the Golgi area the areas with rough ER were also devoid of CA-granules. The ribosomes were evenly scattered through the cytoplasm, frequently forming polysomal aggregates The endoplasmic reticulum possessed a marked tendency to enlarging and large cytoplasmic cisternae were often noted Rather small elongated or ovoid mitochondria were always present, and were frequently grouped on the paranuclear area of cytoplasm. The mitochondrial matrix was usually darker than the surrounding cytoplasm.

Centriols were present and cilia formation occurred frequently A special characteristic of the CA-storing cells seemed to be the multivesicular aggregates which were found particularly in the adrenomedullary cells. Aggregates were usually found as extensions of the cytoplasm, filled with

agranular vesicles or free membrane-bound collections of vesicles, in the intercellular space. Multivesicular bodies were also found regularly

Glycogen particles were scattered evenly through the cytoplasm in the form of alpha particles. Occasionally cytoplasmic areas with only tightly packed glycogen aggregates were found Glycogen could be found at all ages and there were no marked changes in amounts.

Nerves in paraganglia and adrenal medulla

The para-aortic bodies were in closely contact with primitive sympathetic plexus at all stages of development However the supply of paraganglia by nerve fibres remained very sparse during the period studied. After the age of 10 weeks solitary axons or bundles of them were found among the para-aortic tissue.

The nerve fibres often ran parallel with the walls of the blood vessels and probably often ended on the arteriolar walls. Axons were also found between the paraganglionic cells and in intimate contact with the cellular surface. The fibres contained microtubuli, and terminal clubs with agranular vesicles were occasionally found Although axon-terminals were present no typical synaptic contacts between nerve fibres and paraganglionic cells were found in any paraganglia studied

With increasing age the relative density of the axon network increased slowly although it remained sparse. Smaller clusters of granule-containing cells completely or partially surrounded by developing sympathetic ganglionic tissue were frequently found. No synapses were found on the CA-storing cells in these groups either The sympathetic neurons were usually separated from the CA-storing components by a thin layer of connective tissue elements, or only by bundles of neuronal processes. Neurones or neuroblasts were never found within the para-aortic bodies.

Nerve fibres were found within adrenal cortical elements after the 7th week of development. Cross sections of thin axon bundles were found in the central area of adrenals in 8—9 week old fetuses. The primitive sym

Fig 60 Two synapses facing to neighbouring CA-storing cells in adrenal medulla of 15 week old fetus. Both granular and agranular vesicles were found in the axon terminals. Note the larger and paler type of CA-storing vesicles which probably store adrenalin. (MC=medullary cell, SY=synapse on the medullary cells, PN=preganglionic nerve) 19700 ×

Fig. 61 A synapse on an intracapsular CA-storing cell. Note the typical invagination of the plasmamembrane of the CA-storing cell. Processes of sympathetic neuroblasts surround this intracapsular CA-storing cell. This synapse was probably formed by



axonal processes from sympathetic neuroblasts in the middle of the same collection of primitive sympathetic elements. (MC=mast Marr cell, SN=sympathetic neuroblast, SY=synapse.) 38800 ×

pathetic cells invaded the cortex following the course of nerves. Thus, in 10 weeks old fetuses, wherever a cluster of primitive sympathetic cells was sectioned serially nerve trunk was found either between the cells or passing by sending branches to the group In 10 weeks old fetus nerve fibres were often, but not invariably found in the vicinity of the developing granule containing cells.

The structure of the first intra-adrenal axons was primitive A few irregularly organised neurofibrils were found inside the axonal membrane. Neurotubules were identified after the 10th week, when occasional dense cored vesicles (diameter 1100Å) were also met. The Schwann elements were regularly found around the larger trunks after the 10th week (Fig 57)

For studying the development of synaptic contacts groups of medullary cells and groups of primitive sympathetic cells were sectioned serially During the 10th—11th weeks the number of axons in intimate contact with CA-storing cells markedly increased and a few agranular vesicles, diameter about 450 Å, were met with in the slightly enlarged areas of the axons. During the 11th and 12th weeks the number of enlargements in axons running between the medullary cells clearly increased, and more agranular vesicles could be found in the terminal enlargements of the axons (Fig 62) In a 12 week old fetus, typical synaptic profiles were observed in addition to the free axon terminals without axo-somatic con tacts or synaptic thickenings.

The presynaptic enlargement contained two types of vesicles. Agranular vesicles with a diameter of c. 460 Å usually collected on the central region of the terminal with a membrane contact on the synaptic thickening CA storing granular vesicles with mean diameter of c 980 Å. The number of granular vesicles increased slowly with age. The relation of the numbers of agranular vesicles to dense-cored ones was c. 30 at 12 weeks, decreasing with age, and being c. 10 at 16 weeks.

The synapses were found only on the mature medullary cells. The axons usually ended on the surface of the cells without indenting the plasma membrane. The nerve fibres often ran between the tightly packed granule-containing cells, and formed the synaptic contact in the middle of the group In adrenals of 12 and 13 week old fetuses only a few solitary nerve endings could be found in a medium sized group of medullary cells (consisting of 8—10 cells) However more nerve endings developed with the increase of the amount of granule containing cells. The original relation between the number of synapses and number of granule-containing cells increased clearly Instead of only solitary endings, 2 or three synapses



Fig. 61. Higher magnification of two axon terminals on dromomedullary CA-storing cells. 86440 ×

facing in different directions were often met grouped together fetuses older than 15 weeks. A single nerve ending sometimes formed synapses with at least two chromaffin cells, but this was not a general feature. Connective tissue elements such as collagen fibres often accompanied the axons in the groups of medullary cells but the cells of Schwann were rarely seen between the medullary cells.

It is probable that new mature medullary cells appeared through mitotic division of immaturer forms and by differentiation of the medullary patterns of primitive sympathetic tissue. The latter variety were called "intracapsular medullary cells" because of their localization inside the thin connective tissue layer surrounding the collections of primitive

pathetic cells invaded the cortex following the course of nerves. Thus, in 10 weeks old fetuses, wherever a cluster of primitive sympathetic cells was sectioned serially nerve trunk was found either between the cells or passing by sending branches to the group In 10 weeks old fetus nerve fibres were often, but not invariably found in the vicinity of the developing granule containing cells.

The structure of the first intra-adrenal axons was primitive. A few irregularly organised neurofibrils were found inside the axonal membrane Neurotubules were identified after the 10th week, when occasional dense cored vesicles (diameter 1100Å) were also met The Schwann elements were regularly found around the larger trunks after the 10th week (Fig 57)

For studying the development of synaptic contacts groups of medullary cells and groups of primitive sympathetic cells were sectioned serially During the 10th—11th weeks the number of axons in intimate contact with CA-storing cells markedly increased and a few agranular vesicles, diameter about 450 Å, were met with in the slightly enlarged areas of the axons. During the 11th and 12th weeks the number of enlargements in axons running between the medullary cells clearly increased, and more agranular vesicles could be found in the terminal enlargements of the axons (Fig. 62) In a 12 week old fetus, typical synaptic profiles were observed in addition to the free axon terminals without axo-somatic contacts or synaptic thickenings.

The presynaptic enlargement contained two types of vesicles. Agranular vesicles with a diameter of c 460 Å usually collected on the central region of the terminal, with a membrane contact on the synaptic thickening. CA storing granular vesicles with mean diameter of c. 980 Å. The number of granular vesicles increased slowly with age The relation of the numbers of agranular vesicles to dense-cored ones was c. 30 at 12 weeks, decreasing with age and being c 20 at 16 weeks.

The synapses were found only on the mature medullary cells. The axons usually ended on the surface of the cells without indenting the plasma membrane. The nerve fibres often ran between the tightly packed granule-containing cells, *and formed the synaptic contact in the middle* of the group In adrenals of 12 and 13 week old fetuses only a few solitary nerve endings could be found in a medium sized group of medullary cells (consisting of 8—16 cells) However more nerve endings developed with the increase of the amount of granule containing cells. The original relation between the number of synapses and number of granule-containing cells increased clearly Instead of only solitary endings, 2 or three synapses

of the synapses on intracapsular medullary cells were formed by processes of sympathetic neuroblasts. The processes of the developing neurons did not grow alone outside the boundaries of primitive sympathetic cell groups. The synapses on extra-capsular medullary cells were formed by axons from the preganglionic trunks (Fig. 63)

The nature of the synapses was also studied using potassium permanganate fixation. The agranular vesicles in the axon terminals remained empty after this fixation as well.

Discussion

Differentiation of primitive sympathetic cells

The ultrastructural changes in primitive sympathetic cells were studied. Slight variations in cellular size and shape were usual before the first signs of catecholamine synthesis appeared. However no specific changes of the cytoplasmic components occurred before the empty vesicles were filled with electron dense material. The filling was gradual and several degrees of density of the materials were found during the 9th—10th weeks. The first CA-granules appeared scattered throughout the cytoplasm and thus no light was shed on the site of formation of these vesicles.

A well developed Golgi apparatus was typical of the early chromaffin cells at all stages and the vesicular part of it was well developed. Elfvin (1966) suggested that the vesicular membrane might possibly have been formed in the Golgi apparatus, and assumed the concentrating ability of catecholamines just before release from the apparatus.

The present results suggest that the ability to concentrate catecholamines is aquired later when the vesicles were scattered through the cytoplasm. Moderate electron dense material was occasionally found between the lamellae of Golgi apparatus, but the first CA-granules showed no special relations to it. This is compatible with the findings of Coupland & Weakley (1968) who found no relationships between these structures.

Formaldehyde induced fluorescence was regularly present in the developing paraganglionic tissue after the 8th week of development. However no specific ultrastructural changes could be demonstrated in fetuses younger than 8 weeks. This suggests the presence of cytoplasmic catecholamine stores other than granular vesicles. It is also possible that the catecholamines (noradrenaline) in the actual stages of cellular differentiation, although bound in the vesicles, could not be fixed by the means used.

The difference between the appearance of FIF and fine structural signs of catecholamines questions the values of electron microscopy in localization of small amounts of catecholamines. Later however when larger quantities of CA are involved, correlations evidently exist between the amount of CA-granules and CA amounts stored (Van Orden *et al* 1970) On the other hand, the variations of FIF did not follow the changes in CA content of mature CA-storing cells unless considerable quantities were released from the cell.

The signs of catecholamine synthesis were first noted in the extra-adrenal collections of primitive sympathetic cells. In the 8 weeks old specimens, CA-granules could not be demonstrated in the intra-adrenal primitive sympathetic cells. This initial difference disappears during the 10th—12th week. The initial delay of differentiation of intra-adrenal CA-storing elements was not observed in the rabbit adrenals (Coupland & Weakley 1968)

Whether intra or extra-adrenally these first CA-storing cells were close to the primitive capillaries. The relations of these cells to the circu lation was studied from serial sections. Wherever developing CA-storing cells were found within the primitive sympathetic tissue capillaries could be found in the vicinity

The most prominent and specific feature of the cytoplasm of devel oping paraganglia and adrenal medulla was the presence of variable amounts of granular vesicles which probably store catecholamines. The size of the vesicles increased from the initial 800—1200 Å to 1300—3000 Å in the mature cell. No marked differences could be demonstrated between the granular size of CA-granules of paraganglionic and adrenomedullary CA-storing cells. However after the 16th week, larger and paler CA-granules measuring 2400—4300 Å were found in the adrenomedullary cells in addition to the former variety This change from a smaller granule with electron dense core to a larger one with paler core occurred simultaneously in a great proportion of the cells. The larger granules were not dominant in any cell. Cells with only a smaller variety of CA-granules were also found at all stages.

The smaller CA-granules were probably storing noradrenalin and the larger generation might represent the adrenaline storing type described by several authors (e.g. Coupland & Hopwood 1966) However the division into two generations of granules was not strict instead, granules with intermediate properties were also found in cells with large A type vesicles. In paraganglia an intermediate type of vesicles was frequently met but the lack of typical adrenaline storing CA-granules was evident. Al-

though correlations between the granular structure and presence of adrenalin in chromaffin cells have been demonstrated, slight variations in the electron density and granular size do not inevitably indicate the presence of A (Van Orden *et al.* 1970). Thus it is probable that the secondary amine was not stored in the adrenal medullary cells in granules until after the 16th week. The type of the majority of CA-granules in older fetuses also suggested the presence of NA. In paraganglia only the noradrenaline-type was identified with certainty. These observations are compatible with the pharmacologic estimations of catecholamines (Shephard & West 1952, Hunter *et al.* 1953, Niemineva & Pekkarinen 1953, Coupland 1953, Brundin 1966). All these previous authors were able to demonstrate only traces of adrenaline in fetal adrenals and solely noradrenaline in the paraganglia. More recently von Studnitz (1968) and Gennser & von Studnitz (1969) studied the CA-contents of human fetal paraganglia and adrenals with more modern methods. Equal concentrations of NA and A were found in adrenals and traces of A were also present in the paraganglia. The present results hence suggest that the intermediate form of CA-storing granules may also be concerned with the storage of A. This type of CA-granules was present in both paraganglionic and adrenomedullary CA-storing cells. The changes in the internal structure of the granules from the uniform very electron dense to paler one with reticular or granular substructure may be a sign of changed storage or synthesis patterns.

Similar catecholamine storing granules have been described in CA storing elements of sympathetic ganglion by several authors (Siegrist *et al.* 1966, 1968, Matthews & Raisman 1969, Williams & Palay 1969, Van Orden *et al.* 1970). The cells invariably contained large amounts of catecholamines; this was verified by other methods. It was suggested that this generation of large CA-granules should be considered as cytoplasmic organelles required for stable storage of CA. The internal structure, the density of the core and its possible substructures could be dependent on the quality and quantity of the amine stored. Thus, the intermediate form of CA-granules could represent mixed storage of primary and secondary CA, NA and A. The elongated forms of CA-granules often found in both adrenal medulla and paraganglia were considered as indicators of the insignificance of granular shape. The presence of increasing amounts of A-storing granules in the cytoplasm of mature CA-storing cells indicated that the cellular metabolism probably slowly synthesized A from the primary amine.

According to Coupland (1965) the adrenal medullary cells develop much more slowly than those in the pre-and para-aortic region. Furthermore primitive sympathetic cells persist in the human adrenal gland up to birth, forming large groups associated with nerve fibres. This basic organization was reported by Keene & Hewer (1927) Crowder (1957) Ito (1959) and Boyd (1960) at light microscopic level.

The differentiation of extra-adrenal CA-storing cells was rapid after the 9th week and at the age of 12 weeks the majority of cells showed characteristics of mature CA-storing cells. The persisting large groups of primitive sympathetic elements in adrenal medulla provided good conditions for studying the further differentiation. Since neurones are a constant feature of the human adrenal medulla (Coupland 1965) the general properties of developing sympathetic neurons, previously described by Pick *et al* (1964) were specially considered.

The cellular organisation of the fetal premedullary area was clear The anastomosing cords and trunks of the fetal cortex and wide capillary sinusoids formed the base The medullary elements could be divided into two groups (1) numerous solitary small groups of CA-storing cells lying on the walls of capillary sinusoids (2) large tightly packed and avascular groups of primitive sympathetic cells, also surrounded by cortical cells and blood vessels. It seems probable that the differentiation proceeded faster in the smaller groups, and the first mature CA-storing cells found intra-adrenally after the 11th week were in these small groups on the vessels. On the boundaries of primitive sympathetic groups the intracapsular variety of CA-storing developed, as a result of differentiation of more primitive forms. However only a few intermediate forms were met in this situation, indicating that the characteristics of mature CA-storing cells were rapidly assumed once the differentiation started.

Sympathetic neuroblasts were also found within the groups of primitive sympathetic cells. Their cytoplasmic processes were followed, and the possibility of postganglionic innervation of a proportion of chromaffin structures was suggested (Fig 63) The course of the processes of medullary neurones is obscure (Coupland 1965) Lewis & Shute (1969) stated that they are postganglionic sympathetic neurones whose axons join the medullary plexus at least in the rat The present results support this conception but in addition, the possibility of direct postganglionic innervation of a minor part of medullary cells should be considered (Fig 63)

Mitotic figures were frequently met within the primitive sympathetic

Schematic representation of possible postganglionic innervation in human fetal adrenal medulla

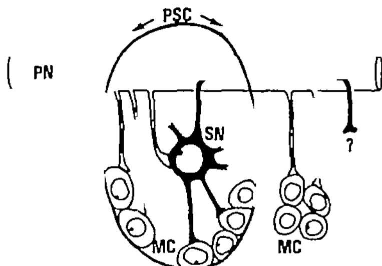


Fig. 63 Schematized primitive sympathetic cell group (PSC) with intra- and extra-capsular medullary cells (MC). Sympathetic neuroblast (SN) described in the middle of the primitive sympathetic elements.

Preganglionic nerve trunk (PN) send branches to medullary cells and sympathetic neuroblasts forming synapses on both cell types. Sympathetic neuroblasts probably form postganglionic contact directly with the intracapsular medullary cells or send axons with the preganglionic nerve trunk.

cell-groups, but were rare in the collections of maturer medullary cells. Cells with large numbers of CA-storing granules were only occasionally observed to undergo mitotic division. It is probable that the slow growth and condensation and development of adrenal medulla is at least partly due to the evidently slow production of new medullary cells by the groups of primitive sympathetic elements. In the older fetuses particularly mitoses were found only in these groups.

The persistence of primitive cellular elements as isolates among rapidly differentiating cells of the same origin is discussed later.

Innervation of paraganglia and adrenal medulla

Although nerve fibres and occasional terminal enlargements of axons were found in the paraganglia, no synaptic contacts could be demonstrated. The nervous network was sparse during the period studied. The findings were in agreement with the results of Coupland *et al.* (1968) who did not find synapses in the rabbit paraganglia. Neither did Viragh *et al.* (1966)

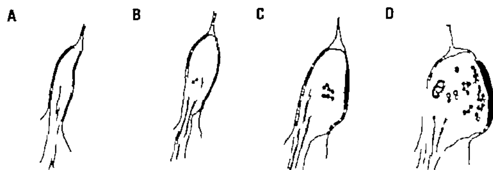


Fig 64 Schematic representation of the development of a synapse on the adrenomedullary CA-storing cell. The terminal enlargement of the presynaptic axon with a few synaptic vesicles were demonstrated before the thickenings of the plasma membrane.

report any axosomatic contacts in the mouse paraganglia. In the only previous EM work on human fetal paraganglionic tissue Battaglia (1969) did not comment on the possible innervation Hence it seems probable that the human fetal paraganglia lack functional innervation at least during the first two trimesters of pregnancy The presence of nervous network and occasional axon terminals, however might indicate some kind of nervous function in paraganglia possibly a sensory one

After the 12th week, synapses were constantly found on the adrenomedullary cells. The nerve supply was effective and the synapse cell ratio was high. The structure of the synapses was typical of the cholinergic nerve endings. Agranular vesicles with mean diameter of 460 Å and granular vesicles with mean diameter of 980 Å were found. A schema of the development of a synapse on adrenal medullary cells is presented in Fig 64 The initial synaptic profiles were identified between the chromaffin cells as enlargements of axons containing a few agranular vesicles. A slight increase in the electron density of the synaptic membranes was only occasionally noticed at this stage. Constant accumulation of electron dense material was not found prior to the increase of amounts of agranular vesicles in the terminal This finding disagrees with the idea proposed by Tennyson (1969) that the synaptic thickening may develop before the accumulation of synaptic vesicles occurs. However the only electron microscopic data on human fetal sympathetic tissue available was the study of Pick *et al.* (1964) where no developmental comments were presented. Although the sequence of events in the development of synapses in the central nervous system of vertebrates may be the opposite, synaptic thickenings in the developing synapse on human fetal CA-storing cell appeared simultaneously or after the accumulation of synaptic vesicles (Fig 64)

The nature of the synaptic vesicles was tested using potassium permanganate fixation (Hökfelt (1969) The vesicles remained empty in these specimens, thus indicating the absence of CA in the nerve terminals.

In vitro electrical stimulation experiments (for ref see Tennyson 1969) have shown that the onset of bioelectric activity suggestive of synaptic transmission correlates closely with the earliest point at which synaptic profiles could be demonstrated in the tissue culture Hence the mature synaptic profiles found in increasing amounts in human fetal adrenal medulla strongly suggest that a synaptic form of nervous transmission may be functional after the first trimest r of pregnancy CA are probably secreted from the adrenal medulla after stimulation of the preganglionic innervation of the gland However the general functional state of the sympathetic nervous system during fetal life is not known.

CHAPTER V

GENERAL DISCUSSION

On the differentiation of CA-storing cells

Lempinen (1964) discussed widely the possibilities of humoral especially adrenocortical regulation of the differentiation of rat chromaffin tissue. Since then several problems of fetal endocrinology have been settled including the effect of adrenocortical steroids on the synthesis of adrenaline from noradrenaline (Eränkö Lempinen & Räisänen 1966 Coupland & McDougall 1966) Wurtman & Axelrod (1966) found that the activity of the enzyme phenylethanolamine-N methyl transferase was regulated by the presence of cortical hormones, especially hydrocortisone This has been more recently confirmed by Roffi (1968 a b) Thus, the last stage in the differentiation of chromaffin cells was under hormonal control. On the basis of the present results it is also suggested that the humoral factors are important when the direction of differentiation of primitive sympathetic elements is determined. The data are summarized

(1) The first signs of further differentiation of primitive sympathetic cells were accompanied by primitive vascular supply of the same region.
(2) The areas of developing paraganglia obtained an effecient vascular supply at all stages of development
(3) The poorly vascularized areas of primitive sympathetic patterns in the para-aortic region showed no changes towards CA-storing granule containing cells sympathetic neuroblasts were identified instead.
(4) The intra-adrenal groups of primitive sympathetic cells were completely avascular and also showed delayed patterns of differentiation by comparison with other primitive sympathetic elements. The boundaries of the groups were surrounded by cortical tissue or venous sinusoids of the premedullary area. CA-storing granule containing cells differentiated on the peripheral zone of the cell groups, and in the central parts slow differentiation towards sympathetic neuroblasts could be observed.

The vascular contacts of differentiating CA-storing cells were well developed at all stages of development and in all localizations studied.

Conditions for humoral stimulation of differentiation were suitable

The onset of steroidogenic activity in human fetal adrenal gland has not been studied so widely as the characteristics of midterm steroid metabolism. Johannison (1968) followed the differentiation of human fetal adrenal cortex and found that the ultrastructural markers of steroidogenic activity were present in the innermost layer of fetal cortex at the age of 7—8 weeks. This was partly in agreement with the previous studies of Bloch (1967) and Villee (1961). In the incubation studies of Saure and Timonen (personal communication) steroidogenic activity was found in adrenals of 7—8 weeks old human fetuses.

These findings together with further knowledge on fetoplacental endocrinology indicate that steroid hormones were present in fetal tissues, and probably also in the circulation, during the time when the chemodifferentiation of CA-storing cells evidently starts. The fetal adrenals are capable of forming all biologically active corticosteroids, from progesterone via cortisone. Although the synthetic pathways of the fetoplacental unit were widely elucidated, the biological effects of the different types of steroids produced remain to be established. Recently evidence for humoral regulation of several differentiational events has been accumulated (For ref. see Jost 1966). It has also been shown that products of fetal endocrine glands are often important when the direction of differentiation is determined (Wells 1966). Most of the steroid metabolites pass the placental barriers with relative ease (Villee 1969), and thus the patterns of the free hormonal steroids of pregnancy are probably also present in the fetal circulation.

It is thus evident that several different steroid metabolites are present in the fetal circulation during the early stages of further differentiation of primitive sympathetic tissue. The present observations on the general organization of vascular supply in the developing primitive sympathetic tissue have led to the conclusion that humoral agents are probably important in the determination of the direction of this differentiation. It is further assumed that some steroid hormone is responsible for the effect.

The close relationship between CA-storing elements and local capillaries has also been reported in localizations other than adrenal medulla and para-aortic bodies. Small intensely fluorescent cells, first identified by Eränkö & Härkönen (1963, 1965) in the cervical ganglion of the rat, have later been studied intensively (Grillo 1964, Siegrist *et al.* 1968, Eränkö & Eränkö 1971 and others). A common feature of the small intensively fluorescing cells was their close relation to capillaries. This might be a sign of a secretory function or might indicate the ability to react to chemical stimuli. A chemoreceptor function for these cells was mentioned by Eränkö & Eränkö (1971). It may however be possible

The physiologic role of the majority of fetal CA-storing tissue the paraganglia is still obscure. Although the high CA content of the paraganglia has been demonstrated by several investigators (for ref. see Coupland 1965) no physiological circumstances have been found under which the CA were discharged into the circulation. Coupland (1953) suggested that the paraganglia may function as a real endocrine organ concerned with the control of vasomotor tone at a time when the adrenal medulla and the sympathetic nervous system are still at a more primitive stage of development.

Comline (1966) came to the conclusion that the mechanism for release of NA from fetal adrenomedullary cells was different to that in adult chromaffin tissue. He suggested a direct effect of oxygen deficiency on the chromaffin cells instead of a stimulation via preganglionic nerves. The observations of Brundin (1966) support the idea of direct effect of chemical stimulus. Experimentally produced strong hypoxia caused a marked loss of CA (NA) from the rabbit para-aortic bodies. Further no effect was found after hypoglycemia, which is known to stimulate the adrenal medulla through the sympathetic nerves. Brundin stated that the paraganglia may function as an endocrine organ in certain pathophysiologic circumstances, such as fetal asphyxia. Muscholl & Vogt (1964) stimulated chromaffin cells which were situated within the abdominal sympathetic ganglion of dog and they also suggested a humoral mechanism for CA release from the paraganglionic tissue. Hence, no evidence for the presence of functional innervation was available after these histophysiologic studies. The light microscopic observations on the innervation of paraganglia (for ref. see Ch. II) could be confirmed by electron microscopy. Coupland (1968, 1970) studied the development of rabbit paraganglia and adrenal medulla and regularly found a sparse network of nerves within the para-aortic bodies. Synaptic profiles were found only on the intra-adrenal chromaffin cells. It was also suggested, after ultrastructural studies, that the paraganglia lack a functional innervation.

When added to the previous information, the present findings led to the following conclusions. (1) The human fetal paraganglia probably function as an organ secreting CA (NA) during fetal life. (2) The mechanism for release of CA from extra-adrenal chromaffin cells is different from that in intra-adrenal chromaffin cells, which were richly innervated.

Hervonen & Korkala (1971 c d) used a perfusion technique modified from the method of Westin (1958) to show the effect of hypoxia on human fetal chromaffin tissue. A clear decrease of specific, formaldehyde induced fluorescence was found after hypoxia in paraganglionic cells. In adrenal

medulla the discharge of CA was not evident. Hence it seems probable that the human fetal paraganglia function as an emergency organ releasing the CA into the circulation after a direct stimulation of chemical (humoral) factors.

The physiological role of CA in fetal circulation has been partly elucidated (for ref see Ehinger et al 1967) Ductus venosus of the human fetus is probably under adrenergic control A contractile response was produced by injections of CA (NA and A) Hence the blood flow in the umbilical cord may be regulated by the adrenergic mechanism Aronson et al (1970) suggested that the closure of ductus arteriosus of human fetuses may also be controlled by the adrenergic mechanisms. NA produced a contractile response in the vessel. These two important sphincter mechanisms of human fetal circulation may possibly react to the CA (NA) discharge from the paraganglia. The fetal CA storing tissues, both extra and intra-adrenal, might be of functional importance when disturbances of fetal homeostasis are to be compensated

The functional status of the sympathetic nervous system during the early stages of fetal life is not known The morphologic maturity of innervation of developing adrenal medulla provided possibilities for nervous regulation of CA release Hence the fetal adrenal medulla although representing the minor part of fetal CA-stores, might be of functional importance in regulation of the vasomotor tone of fetal circulation under physiologic conditions.

SUMMARY

The development of human fetal abdominal paraganglia and adrenal medulla was studied using four different methods. Twohundred fetuses aged 6—27 weeks were examined.

Chapter I Formaldehyde induced fluorescence

Green FIF probably due to noradrenaline was demonstrated in cells of the primitive sympathetic tissue of 7 weeks old fetus. Two weeks later fluorescing cells were also found within adrenal cortical elements. New fluorescing cells appeared during the 8th—9th weeks through the whole length of the abdominal aorta soon forming rounded or ovoid clusters, primitive paraganglia. The size of the paraganglia the intensity of the FIF and the total amount of paraganglionic tissue increased rapidly during the third month of fetal age.

The adrenal medulla of 9 weeks-old fetus consisted of small groups of fluorescing cells scattered evenly among the trabeculae of cortical tissue. The amount of medullary cells increased rapidly during the third month and new cells differentiated from the primitive sympathetic elements of the gland. The primitive sympathetic cells formed large groups in the central areas of the cortical tissue. After the age of 14 weeks, a weak greenish fluorescence was constantly found in several cells inside these groups. The collections of primitive sympathetic elements were surrounded by a rim of intensively fluorescing medullary cells.

All fluorescence observed in the developing CA-storing elements was probably due to noradrenaline The developing neurones in abdominal sympathetic ganglia showed weak green FIF after the 14th week of fetal life. Small clusters of brightly fluorescing CA-storing cells, possibly SIF cells, were occasionally found within the ganglion.

Chapter II Cholinesterases

The primitive sympathetic trunk of 8 weeks old fetus was AChE positive Finer fibers reached from this trunk to the adrenal cortical

elements. An adrenomedullary anastomosing network of finer fibers developed during the 8th—9th weeks of fetal life. Rich branching occurred between the primitive sympathetic cells which formed large groups along the course of the AChE positive fibres. After the 12th week of fetal age fine end fibers were found in the same localization where groups of CA storing cells were demonstrated. Cellular profiles indicating cytoplasmic activity were not found. The tightness of the adrenomedullary network increased with age and a clearly restricted innervated medulla was observed in the older fetuses.

The paraganglia were completely negative up to the 12th week of fetal life. After this age fine AChE positive fibres were found to invade the paraganglionic tissue from the strongly positive preaortic sympathetic nerve trunk. The fibres formed a loose and irregular network which extended after the fourth month through the entire paraganglionic body.

The developing sympathetic neurons of the abdominal ganglia showed weak AChE positivity after the third month of fetal life.

The nsChE was intra-adrenally found in the form of coarse trunks passing towards the center of the gland from the sympathetic plexus. Finer fibres, which were AChE positive were not found after the demonstration of nsChE. The mature adrenomedullary cells were occasionally nsChE-positive in the older fetuses, but negative cell groups dominated.

A strong reaction surrounded the groups of primitive sympathetic cells. Coarse short streaks of activity were found scattered through the medullary area corresponding to the distribution of CA storing cells.

The nsChE activity in paraganglia was exhibited by the supporting elements and formed an even reticulum between the collections and cords of paraganglionic cells. Later after the third month, a weak cytoplasmic positivity of the CA-storing cells became a constant feature.

Chapter III Vascular supply

The relations of developing and mature CA-storing cells to the microcirculation both in the paraganglia and in adrenal medulla, were intimate at all stages of differentiation. The capillaries were present in the primitive sympathetic tissue when the first signs of further differentiation on the "CA-storage line" could be observed. Later wherever collections of CA storing cells were found the typical rich sinusoidal capillary network was always present. The sinusoidal organization of capillaries in the paraganglia bears a strong resemblance to the microcirculatory patterns of endocrine glands.

The intra-adrenal sinusoidal system was well developed during the period of fetal life studied. The central sinusoids widened especially during the third and fourth month. A direct arterial supply of the medullary sinusoids developed during the third month. The medullary CA-storing cells were situated on the capillary sinusoids with close contact to the vessels. The collections of primitive sympathetic cells were completely avascular but effectively surrounded by sinusoids.

Chapter IV Fine structure

The developing paraganglionic and adrenomedullary CA-storing cells were found to exhibit similar features. The first signs of further differentiation of primitive sympathetic cells were found in the preaortic area of 8 weeks old fetus. The filling of the previously empty cytoplasmic vesicles with electron dense material became a regular observation after the 9th week of fetal age. The size of these dense cored vesicles, which probably store CA, increased during the 9th—19th week from 1005Å to 2590Å (mean diam.) No marked differences between mature paraganglionic and adrenomedullary CA-storing cells could be found.

Two main pathways for further differentiation of primitive sympathetic cells were found. The cells in close contact with the capillary sinusoids or cortical cells developed to CA-storing medullary cells. On the other hand, cells in the centre of the large avascular groups of primitive sympathetic elements assumed characteristics of sympathetic neuroblasts.

The CA-storing cells of fetuses younger than 16 weeks contained only NA type of CA-granules but after this age A-type of granules were also found in the adrenomedullary CA-storing cells. An intermediate form between the main types was found in both paraganglia and adrenal medulla.

The paraganglionic CA-storing cells were not innervated. Free axon terminals were occasionally found in the paraganglia but typical synaptic profiles were not met. The adrenomedullary cells, instead, were richly innervated after the third month of fetal life. Synaptic contacts between the preganglionic axons and CA-storing cells were morphologically mature after the 14th week of fetal life. Synapses were also found on the sympathetic neuroblasts.

A schematic representation of the probable course of processes of the sympathetic neuroblasts was presented.

The development of synapses on CA-storing cells was specially studied. The axon terminals with agranular synaptic vesicles developed prior to the membrane thickenings of the pre- and postsynaptic membranes.

Chapter V General discussion

The main characteristics of differentiation of human fetal CA-storing cells were summarized. The possibility of humoral induction of differentiation of CA-storing cells was discussed in the light of recent data on the effect of glucocorticoids on the primary differentiation. It was suggested that the humoral agents, possibly glucocorticoids, could be the agents which direct the differentiation of primitive sympathetic cells towards the "CA-storage-line". The close contact of primitive sympathetic cells to the microcirculatory patterns was essential for this differentiation. The existence of "SIF"-cells in the sympathetic nervous system was discussed on the basis of the humoral regulation hypothesis.

The evidence indicating endocrine activity of fetal CA-storing tissue and especially paraganglia was summarized. The present data together with earlier observations on the nature of paraganglia in different mammalian species led to the conclusion that the human fetal paraganglia evidently function as an active endocrine organ. The mechanism of discharge of products from the paraganglia might be different from the mechanism functioning in the adrenal medulla which is richly innervated. On the basis of recent experimental data of the present author it was suggested that the extra-adrenal CA-storing elements together probably act as an emergency organ discharging noradrenaline in certain pathophysiological conditions of fetal life. The paraganglionic secretion is probably regulated by direct cellular effect of chemical or humoral agents.

REFERENCES

Aronson S., Gennser, G., Owman Ch. & Sjöberg N-O Innervation and contractile response of the human ductus arteriosus. European Journal of Pharmacology 1970 *11* 178—186.

Bakay L. von: Das chromaffine System der Harnblase des Menschen, mit besonderer Berücksichtigung der Innervation. Z mikr-anat. Forsch. 1938, *43* 131—142.

Battaglia, G Ultrastructural observations on the biogenic amines in the carotid body and aortic-abdominal bodies of the human fetus. Z Zellforsch. 1960 *99* 529—537

Björklund A., Enemar, A. & Falck, B.: Monoamines in the hypothalamohypophyseal system of the mouse with special reference to the ontogenetic aspects. Z. Zellforsch. 1968 *89* 590—607

Bloch, E.: In vitro steroid synthesis by gonads and adrenals during mammalian fetal development. Exerpt medica internat Congress series 1967 *132* 673.

Brundin T Studies on the preaortal paraganglia of newborn rabbits. Acta physiol. scand. 1966, Suppl. 290 70

Brundin T & Nilsson S E. G.: Osmiophilic granules in preaortal paraganglia of rabbits. Acta physiol. scand. 1965, *65* 27—28.

Boyd J D Origin, development and distribution of chromaffin cells In adrenergic mechanisms, 1960 63—6., Churchill, London.

Champlain J de Malfors T Olson L. & Sachs, Ch.: Ontogenesis of peripheral adrenergic neurons in the rat pre- and postnatal observations. Acta physiol. scand. 1970 *80* 276—288.

Comline, R. S. & Silver, M The development of adrenal medulla of fetal and newborn calf. J Physiol. (London) 1966, *183* 305—340

Corrodi H. & Hillarp N A.: Fluorescenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 1 Identifierung der fluoreszierenden Produkte aus Modellversuchen mit 6, Dimethoxyisochinolinderivaten und Formaldehyd. Helv Chim. Acta. 1963, *46* 2425.

Corrodi H. & Hillarp N A. Fluorescenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 2. Identizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. Helv Chim. Acta. 1964, *47* 911.

Corrodi H. & Jonsson G Fluorescence methods for the histochemical demonstration of monoamines. 4 Histochemical differentiation between dopamine and noradrenaline in models. J Histochem. Cytochem. 1965a, *13* 484.

Corrodi H. & Jonsson B. Fluorescenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 5. Identifizierung des fluoreszierende Produktes aus Modellversuchen mit 5 Methoxytryptamin und Formaldehyd. Acta histochem. 1965b *22* 247

Corrodi H. & Jonsson G The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines. A review on the methodology J Histochem. Cytochem. 1967 *15* 65.

COUPLAND, R. E.: The prenatal development of the abdominal para-aortic bodies in man. J. Anat., Lond. 19 .., 86 35 —72.

COUPLAND, R. E.: On the morphology and adrenaline-noradrenaline content of chromaffin tissue. J. Endocrin. 1953, 9 194—203.

COUPLAND, R. E.: The Natural History of the Chromaffin Cell. Longmans, London, 1965.

COUPLAND, R. E. & HOPWOOD, D.: The mechanism of differential staining reaction for adrenaline and noradrenaline-storing granules in tissues fixed with glutaraldehyde. J. Anat. 1966, 100 227— 13.

COUPLAND, R. E. & MAC DOUGALL, J. D. B.: Adrenaline formation in the noradrenaline storing cells in vitro induced by corticosterone. J. Endocrinol. 1966, 36 317—3..1

COUPLAND, R. E. & WEAKLEY BRENDA S.: Developing chromaffin tissue in the rabbit: an electron microscopic stud. J. Anat. 1968, 10 3 pp. 4..3—433.

COUPLAND, R. E. & WEAKLEY BRENDA, S.: Electron microscopic observation on the adrenal medulla and extra-adrenal chromaffin tissue of postnatal rabbit. J. Anat. 1970, 106 .., pp. 13—231.

CROWDER, R. E.: The development of the adrenal gland in man with special reference to the origin and ultimate location of cell types and evidence in favor of the cell migration theory. Cont. Embry. l. Carneg. Inst. 1957, 36 195— 10.

DICZFALUSY, E.: Steroid metabolism in the fetoplacental unit. In the foeto-placental unit, Pecile A. & Finzi C., Excerpta medica foundation, Amsterdam, 1969.

EHINGER, B., GENNSER, G., OWMAN, CH., PERSSON, H. & SJÖBERG, N-O.: Histochemical and pharmacological studies on amine mechanisms in the umbilical cord, umbilical vein and ductus venosus of the human fetus. Acta physiol. scand. 1968, 2 13— 4.

ELFVIN, L-G.: The fine structure of the cell surface of chromaffin cells in the rat adrenal medulla. J. Ultrastructure Research 1965, 12 263—286.

ELFVIN, L-G.: The development of secretory granules in the rat adrenal medulla. J. Ultrastructure Research 1967, 17 45—62.

ELFVIN, L-G.: A new granule-containing nerve cell in the inferior mesenteric ganglion of the rabbit. J. Ultrastructure Res. 1968, 22 37—44.

ENEMAR, A., FALCK, B. & HÅKANSON, R.: Observations on the appearance of norepinephrine in the sympathetic nervous system of the chick embryo. Develop. Biol. 1965, 11 268—283.

ERÄNKÖ, O.: On the histochemistry of the rat adrenal medulla. Acta physiol. scand. 1951 ..3 supp. 89.

ERÄNKÖ, O.: On the histochemistry of the adrenal medulla of the rat, with special reference to acid phosphatase. Acta anat. 1952, 16 supp. 17.

ERÄNKÖ, O.: Distribution of fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of the hamster. Acta endocrinol. 1955, 18 174.

ERÄNKÖ, O.: Specific demonstration of acetylcholinesterase and nonspecific cholinesterase in the adrenal gland of the rat. Histochemie 1959, Bd. 8, 237—267.

ERÄNKÖ, O.: Cell types of the adrenal medulla. Ciba Found. Symp. on Adrenergic Mechanisms (Eds. J. R. Vane, G. E. W. Wolstenholme and M. O'Connor). 1960. Churchill Ltd. pp. 103—108.

ERÄNKÖ, O.: Personal communication acknowledged in Falck and Torp (1961).

ERÄNKÖ, O.: Histochemical demonstration of catecholamines by fluorescence induced by formaldehyde vapour. J. Histochem. Cytochem. 1964, 12 487.

REFERENCES

ARONSON S., GENNSER, G., OWMAN CH. & SJÖBERG N-O: Innervation and contractile response of the human ductus arteriosus. European Journal of Pharmacology 1970, *11* 178—186.

BARAY L. VON Das chromaffine System der Harnblase des Menschen, mit besonderer Berücksichtigung der Innervation. Z mikr-anat. Forsch 1938, *43* 131—142.

BATTAGLIA, G.: Ultrastructural observations on the biogenic amines in the carotid body and aortic-abdominal bodies of the huma fetus. Z Zellforsch. 1969 *89* 520—537

BJÖRKLUND, A., ENEMAR, A. & FALCK, B Monoamines in the hypothalamohypophyseal system of the mouse with special reference to the ontogenetic aspects. Z Zellforsch. 1968, *89* 590—607

BLOCH, E. In vitro steroid synthesis by gonads and adrenals during mammalian fetal development. Exerpt medica internat Congress series 1967 *132* 675

BRUNDIN T Studies on the preaortal paraganglia of newborn rabbits. Acta physiol. scand. 1966, Suppl. 290 0.

BRUNDIN T & NILSSON S E. G.: Osmiophilic granules in preaortal paraganglia of rabbits. Acta physiol. scand. 1965, *63* 2 —*78.

BOYD J D Origin, development and distribution f chromaffin cells In drenergic mechanisms, 1960 63—82, Churchill, London.

CHAMPLAIN J DE, MALFORS, T OLSON L & SACHS, CH. Ontogenesis of peripheral drenergic neurons in the rat pre- ad postnat l observations. Acta physiol. scand. 19 0 *80* 276—288.

COMLINE, R. S. & SIL ER, M. The development of adrenal medulla of fetal and newborn calf J Physiol. (Londo) 1966, *184* 303—340

CORRODI H. & HILLARP N A. Fluoreszenzmethoden zur histochemischen Sichtbarmachung on Monoaminen. 1. Identifierung der fluoreszierenden Produkte us Modellversuchen mit 6,7 Dimethoxyisochinolinderivaten und Formaldehyd. Helv Chim. Acta. 1963, *46* 24_5

CORROD H. & HILLARP N A. Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. _ Identizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. Helv Chim. Acta. 1964, *47* 911.

CORRODI H & JONSSON G Fluorescence methods for the histochemical demonstration of monoamines. 4. Histochemical differentiation between dopamine nd noradrenaline in models. J Histochem. Cytochem. 1965a, *13* 484

CORRODI H. & JONSSON B. Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 5 Identifizierung des fluoreszierenden Produktes us Modellversuchen mit 5-Methoxytryptami und F rmaldehyd. A ta histochem. 1965b, *25* 47

CORRODI, H. & JONSSON G. The f rmaldehyd fluorescence method for the histo-hemical demonstration f biogenic monoamines. A review on the methodology J Histochem. Cytochem. 1967 *15* 65.

VILLEE, D. BR., ENGEL, L. L., LORING, J. M. & VILLEE, C. A.: Steroid hydroxylation in human fetal adrenals: Formation of 16 alfahydroxyprogesterone, 17-hydroxyprogesterone and deoxycorticosterone. Endocrinology 1961, 69 334

WILLIAMS, T. H. Electron microscopic evidence for an autonomic interneuron. Nature 1967 214 309—310.

WILLIAMS, T. H. & PALAY, S. L. Ultrastructure of the small neurons in the superior cervical ganglion. Brain Res. 1969 15 17—34

WINCKLER, J. Zur Lage und Funktion der extramedullaren chromaffin Zellen. Z. Zellforsch. 1969 96 490—494.

VIRAGH, SZ. & KORENYI BOTH, A. The fine structure of abdominal paraganglia in the newborn mouse. Act. Biol. Hung. 1967 18(2) 161—179

VON STUDNITZ, W. Catecholamine and adenosine trifosphate content of human faetal adrenals. Scand. J. Clin. Lab. Invest. 1968, 22 185—188.

WOOD, J. B. & BARRNETT, J. R. J. Histochem. Cytochem. 1964, 12 197

WURTMAN, R. J. & AXELROD, J.: Control of enzymatic synthesis of adrenaline in the adrenal medulla by adrenal cortical steroids. J. biol. chem. 1965, 241 2301—2303.

YNTEMA, CL. & HAMMOND, W. S.: The development of autonomic nervous system. Biol. Rev. 1947 22 344—59

ZUCKERKANDL, E.: Über Nebenorgane des Sympathicus in Retroperitonealraum des Menschen. Anat. Anz. 1901, 15 9—10

ZUCKERKANDL, E. The development of the chromaffin organs and of the suprarenal glands. Keibel and Mall's Manual of Human Embryology. U.S.A., Lippincott 1912, 2 157—79

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 369

From the Department of Physiology Karolinska Institutet, 104 01 Stockholm, Sweden

STUDIES ON NEUROTRANSMISSION MECHANISMS IN THE RAT AND GUINEA-PIG VAS DEFERENS

BY

GÖRAN SWEDIN

STOCKHOLM 1971

Kungl. Boktryckeriet P. A. Norstedt & Söner
Stockholm 1971
713171

Apart from some previously unpublished results, the present thesis is based on the following papers, which are being referred to by their arabic numerals

1 Owman, Ch N-O Sjöberg and G Swedin Histochemical and chemical studies on pre and postnatal development of different systems of short and "long" adrenergic neurons in peripheral organs of the rat. *Z Zell forsch* 1971 *116* 319—341

2. Swedin, G Postnatal development of the mechanical response of the isolated rat vas deferens to nerve stimulation. *Acta physiol scand* 1971 (Preprint)

3. Swedin G Comparison of the effects of *a* methyltyrosine on the nor adrenaline stores of different peripheral organs of the rat. *Life Sci.* 1970 9 1249—1259

4 Swedin G Effect of nerve stimulation in vitro on the noradrenaline content of the rat vas deferens in the presence of inhibitors of noradrenaline uptake and synthesis. *Acta physiol scand* 1971 (Preprint)

5. Swedin, G Biphasic mechanical response of the isolated vas deferens to nerve stimulation *Acta physiol. scand* 1971 *81* 574—576.

6. Swedin G Endogenous inhibition of the mechanical response of the isolated rat and guinea pig vas deferens to pre- and postganglionic nerve stimulation. *Acta physiol. scand* 1971 (Preprint)

ABBREVIATIONS USED

ACh = acetylcholine CA = catecholamine(s) ChE = cholinesterase DMI = desmethylimipramine ETA = 5,8 11 14-eicosatetraynoic acid α MPT = α methyl *para*-tyrosine NA = noradrenaline PBA = phenoxybenzamine PG = prostaglandin T = tyrosine.

CONTENTS

†1—71317

I INTRODUCTION

During the last decade the sympathetic innervation of the vas deferens has been subject to extensive studies. One of the reasons for this was the introduction by Huković (1961) of the isolated guinea pig hypogastric nerve-vas deferens preparation, which was predicted to be a suitable model organ for demonstration of adrenergic neurotransmission. Very soon, however, pharmacological studies on this preparation revealed results which did not fit with the expected ones for a "classical" adrenergic neurotransmission. Thus, adrenergic α-blocking agents were found in moderate concentrations to enhance rather than inhibit the nerve-induced mechanical responses of the preparation (Boyd, Chang and Rand 1960). Furthermore, the same authors reported that ChE-inhibitors potentiated the motor response of the vas deferens to nerve stimulation. This potentiation could be abolished by atropine, which did not, however, influence the response as such. Hemicholinium (Rand and Chang 1960) but not hexamethonium (Burnstock and Holman 1960) inhibited this response. Together these findings were interpreted as to support the theory of Burn and Rand (Burn 1960, Burn and Rand 1960) suggesting a nonganglionic cholinergic link to be involved in the release of the adrenergic transmitter.

These reports prompted Sjöstrand to reinvestigate the effect of ganglionic blocking agents on the response of the guinea pig vas deferens to hypogastric nerve stimulation. It was found (Sjöstrand 1962 a) that a number of specific ganglion blockers totally abolished this response. Furthermore, section of the hypogastric nerve (Sjöstrand 1962 b) did not result in any decrease in NA content of the vas deferens, which, together with the pharmacological findings strongly suggested a synaptic relay close to the vas deferens. Since then the existence of such a peripheral ganglionic relay located close to the target organs in the most distal part of the hypogastric nerve and belonging to their sympathetic innervation, has been fully established by use of pharmacological (Bentley and Sabine 1963, Birmingham and Wilson 1963, Ohlin and Strömblad 1963), electrophysiological (Ferry 1963, 1967, Kuriyama 1963) and histochemical (Falck, Owman and Sjöstrand 1965, Owman and Sjöstrand 1965) methods. The same pattern of sympathetic innervation by means of so called *"short" adrenergic neurons** has been found to be a characteristic

* The term *"short" adrenergic neuron* was introduced (Owman and Sjöstrand 1965, Sjöstrand 1965) to describe the special kind of postganglionic neurons having their cellbodies located close to the target organs, in contrast to the ordinary "long" adrenergic neurons with their cellbodies in the pre- or paravertebral ganglia.

feature for the pelvic region, including most of the other internal male genital organs from a variety of species (*cf* Sjöstrand 1965) as well as for part of the female reproductive tract (Brundin 1965 Owman and Sjöberg 1966, Owman Rosengren and Sjöberg 1966, Sjöberg 1967) the trigonum area of the urinary bladder (Hamberger and Norberg 1965) and the urethra (Owman, Owman and Sjöberg 1971) These findings have confirmed the old suggestion of Langley and Anderson (1895) that part of the sympathetic innervation of the pelvic organs has its synaptic relay close to the target organs and not in the inferior mesenteric ganglion, as has been supposed by other investigators (for historical survey of the literature see Sjöstrand 1965 and Sjöberg 1967)

The existence of a ganglionic relay in the hypogastric nerve vas deferens preparation demanded the development of a method for a true *postganglionic* nerve stimulation of the preparation. This was done by Birmingham and Wilson (1963) who stimulated isolated vas deferens preparations by *transmural field stimulation* through two parallel electrodes along the organ. By adjustment of the duration of each pulse, postganglionic nerve fibres within the organ can be selectively stimulated (for further details, see General Methods)

With the aid of this method some of the pharmacological findings on vas deferens were reinvestigated. Thereby it was shown that the postganglionic neurotransmission was not sensitive to hemicholinium (Bentley and Sabine 1963) and could not be blocked, although in some studies reduced by atropine (Bentley and Sabine 1963 Birmingham and Wilson 1963 and others) However the resistance towards moderate concentrations of α-blockers, as well as the atropine-sensitive potentiation caused by ChE-inhibitors, still persisted. The significance of these findings will be considered later in the present work.

During recent years the vas deferens has often been included in different studies concerning peripheral adrenergic mechanisms. The short adrenergic neurons have been found to differ from the ordinary long neurons in several respects. Thus they seem to have a lower *in vivo* sensitivity to reserpine (Owman and Sjoberg 1967 Sjöstrand and Swedin 1968 a) and 6-hydroxydopamine (Malmfors and Sachs 1968) and they react differently to immunosympathectomy (Hamberger *et al.* 1965 Zaimis, Berk and Callingham 1965 Iversen Glowinsky and Axelrod 1966) and to specific ganglion stimulants (Bentley 1968) Their endogenous NA content is not increased after decentralization and not reduced by nerve stimulation (Blakely Dearnaley and Harrison 1968 1970) if not extremely high intensity of stimulation is used (Chang and Chang 1965) Several studies on the vas deferens both *in vivo* and *in vitro* have indicated a rather low rate of NA turnover which is only moderately increased by nerve stimulation (Alousi and Weiner 1966 Laverty and Robert

son 1967 Roth, Stjärne and Euler 1967 Austin Livett and Chubb 1967 a, b Weiner and Rabadjija 1968 a, b Johnson, Thoa and Kopin 1971) Furthermore, isolated transmitter granules from bovine seminal vesicle and vas deferens seem to have different properties when compared to those from splenic nerves (Euler 1966, Euler and Lishajko 1966 Stjärne and Lishajko 1966) The possibility therefore exists, that the short adrenergic neurons form a special entity of the sympathetic nervous system (*cf* Owman and Sjöstrand 1966, Owman, Sjöberg and Sjöstrand 1971)

The present work was performed in order further to investigate some of these divergent properties of the motor innervation of the rat and guinea-pig vas deferens.

A In vitro preparations of rat and guinea pig vas deferens

a. *Field stimulated adult rat and guinea pig preparations* (Papers 4 5 and 6) The animals were killed by a blow on the head and the vasa deferentia were dissected out without the hypogastric nerve and carefully cleaned from loose connective tissue and fat. Single organs were mounted in Tyrode containing perspex baths (volume 5 ml) with two parallel platinum electrodes (8 mm apart) shielded in the wall except for the last 10 mm. The Tyrode solution (composition see below) was aerated with 6.5 % CO in O_2 and the temperature kept constant at 37 C with exception for some experiments in Paper 6.

Nerve stimulation was performed by field stimulation (Birmingham and Wilson 1963) with biphasic pulses of supramaximal voltage 1.0 or 1.5 ms duration and frequencies in the range of ?—50 imp/s for 5 or 30 s at 30 or 60 s interval. Biphasic pulses were used to avoid electrolysis in the Tyrode solution. The stimulation parameters were checked on a Tektronix type 50? A oscilloscope. The postganglionic nature of the stimulation was verified by addition to the bath of hexamethonium (50 µg/ml) which did not influence the nerve-induced contractions of the organs.

The mechanical responses of the preparations were recorded isotonically with a Harvard heart/smooth muscle transducer loaded with 0.2—0.5 g on a Grass Model 7 Polygraph The final amplitude of the contractions sometimes exceeded the limits of the ink pen writer which gave a cut appearance of the curve (*cf* Paper 6) With exception of these cases, the recordings were found to be linear within the range of amplification used and amplitude of contractions achieved.

b. *Field stimulated young rat preparations* (Paper 2)

The animals were decapitated and the vasa deferentia with cauda epididymis carefully dissected out with the connecting part of the urethra under a dissecting microscope 1—5 pairs of organs were tied together as indicated in Fig 1 Paper 2. The total lengths of the preparations were 10—23 mm. The mechanical responses of the preparations were recorded as described above (load 25—100 mg)

From the beginning of these experiments it was obvious that the usual 5 ml bath (see above) used for field stimulation was unsuitable due to interference by the oxygen bubbles with the registration of the mechanical activity of these delicate preparations. Therefore a superfusion bath (Fig 1) was constructed

(Lewander 1970) have indicated that the 1 nitroso-2 naphtol reaction (Waalkes and Udenfriend 1957) is not quite specific for T which might partly explain the relatively high levels of endogenous T encountered in the rat vas deferens when determined with this method (Austin, Chubb and Livett 1967 Papers 3 and 4) This unspecificity seems however to be of less importance in the present work, since the purpose of using the nitrosonaphtol method in Papers 3 and 4 was to check the relative organ concentrations of α MPT rather than to determine the absolute levels of T

Statistical analysis of the biochemical values was performed with standard formulae for calculating mean ± standard error of the mean (s.e.m) and Student's t test for significance of differences.

CO_2 in O_2. The hypogastric nerve was placed on two hooked platinum electrodes (2 mm apart) and stimulated at a distance of about 4 cm from the vas deferens. The contractions were abolished on addition of hexamethonium (50 μg/ml) to the bath. The same stimulation parameters as given in section a. were used. In some experiments (Paper 6) Huković preparations were also mounted in 5 ml field stimulation baths and were stimulated alternatively pre- and postganglionically (via the nerve and by field stimulation respectively). The mechanical responses were recorded as described in section a.

For both rat and guinea pig preparations a Tyrode solution of the following composition was used: NaCl 0.8 %, KCl 0.02 %, $CaCl_2$ 0.02 %, $MgCl_2$ 0.01 %, $NaHCO_3$ 0.1 %, NaH_2PO_4 0.005 % and glucose 0.1 %. The Tyrode solution was prepared daily from two highly concentrated stock solutions, stored at +4° C. These stock solutions were prepared once every week since it was constantly observed that the sensitivity of the vas deferens preparations, especially from the rat, decreased significantly if stock solutions older than 6—7 days were used.

B. Extraction and determination of NA, T and α-MPT

The animals were sacrificed under ether anesthesia, after which the different organs were rapidly taken out, weighed and homogenized by grinding (vas deferens) or with an Ultra Turrax apparatus in 2—15 ml of ice-cold 0.4 N perchloric acid. The homogenates were centrifuged for 15 min at 9000 rpm, the supernatant adjusted to pH 3.8 with 2 N KOH and recentrifuged for elimination of the potassium perchlorate. The samples were adjusted to pH 8.4 with 0.1 N NaOH and passed through alumina columns (0.1—0.6 g Al_2O_3, BDH). The catecholamines were eluted with —6 ml of 0.25 N acetic acid, oxidized with iodine and the NA determined in an Aminco-Bowman spectrophotofluorimeter according to Chang (1964) (Papers 1, 3, 4 and 5). The NA is expressed as μg free base (not corrected for losses during the purification procedures) per g wet tissue weight. The recovery of NA added to random samples and carried through the extraction and oxidation procedures was 84.6 ± 1.4 % (mean ± s.e.m., $n = 18$).

The effluents and redestilled water washings (total volume 40—50 ml) from the alumina columns were collected for fluorimetric determination of the sum of T and α-MPT according to Waalkes and Udenfriend (1957) (Papers 3 and 4). With the present method equal concentrations of T and α-MPT give identical fluorescence values. The α-MPT content of the various organs is calculated as the difference between total T' and endogenous T (Spector, Sjoerdsma and Udenfriend 1965, Brodie *et al.* 1966). Recent observations

(Lewander 1970) have indicated that the 1 nitroso-2 naphtol reaction (Waalkes and Udenfriend 1957) is not quite specific for T which might partly explain the relatively high levels of endogenous T encountered in the rat vas deferens when determined with this method (Austin, Chubb and Livett 1967 Papers 3 and 4) This unspecificity seems however to be of less importance in the present work, since the purpose of using the nitrosonaphtol method in Papers 3 and 4 was to check the relative organ concentrations of α MPT rather than to determine the absolute levels of T

Statistical analysis of the biochemical values was performed with standard formulae for calculating mean ± standard error of the mean (s.e.m.) and Student's t test for significance of differences

A. Morphological observations (Paper I)

The normal development of different kinds of neurons including the post ganglionic sympathetic ones are critically dependent on trophic stimulation by a specific nerve growth factor (*cf* Levi-Montalcini and Angeletti 1968) By treatment of animals immediately after birth with nerve growth factor antiserum, an extensive immunological destruction of the postganglionic adrenergic neurons can be brought about resulting in a marked loss of both their endogenous NA and ability to take up exogenous NA (*cf* Levi-Montalcini and Angeletti 1968) One of the most striking dissimilarities between the short and long adrenergic neurons has been their different reaction to such immunosympathectomy

It has been shown that the short adrenergic neurons and also the adrenal medulla remain largely unaffected by administration of nerve growth factor antiserum to newborn rats (Hamberger *et al* 1965 Zaimis, Berk and Callingham 1965 Iversen, Glowinsky and Axelrod 1966) It was suggested that this discrepancy might be due to a more advanced degree of maturity of the unaffected short adrenergic neurons and adrenal medullary cells at the time of birth, making them more resistant to subsequent antiserum treatment.

The present study was undertaken in order to compare the pre and post natal development of the different systems of adrenergic structures in peripheral organs of the rat by means of the Falck Hillarp histofluorescence method and a comparison of the endogenous levels of NA in the heart and vas deferens during the postnatal development It should be emphasized that the histochemical method used does not permit visualization of adrenergic structures unless they contain certain amounts of CA. This means that no conclusion can be drawn from the present study regarding the actual development of the adrenergic structures as such, but only about the appearance of these structures from the time point when they begin to contain detectable CA. The amount of CA needed for histochemical visualization seems to be very low provided that it is concentrated in specific locations (*cf* Jonsson 1967 de Champlain *et al* 1970) Attempts in the present work to increase the endogeneous CA levels did enhance the intensity of the formaldehyde induced fluorescence but did not make visible any other structures than those already demonstrable in untreated animals, thus making it highly probable that the fluorescence method has detected the majority of the developing adrenergic structures (*cf* de Champlain *et al.* 1970)

At 15 days post coitum (earliest investigated term 23th day) the primordia of the sympathetic chains were well visible in the form of two dorsal segmented columns of small branching cells exhibiting an intense CA fluorescence. In the midline ventrally to these two primordia another column of sympathicoblasts developed this seemed to give rise to the prevertebral ganglia and in its most caudal part, to the short adrenergic neurons supplying the pelvic organs. At the level of the adrenals, small intensely fluorescent chromaffin cells were collected in two bilateral groups which later on became partially enclosed by adrenocortical cells.

Bundles of sympathetic nerves were visible in the periphery of the various organs studied at 19—20 days post coitum A varicose terminal innervation, suggestive of a functional transmission, did not start to appear until, or immediately after birth. The final pattern of innervation was usually reached about one week post partum, and the following development proceeded largely in the form of a quantitative increase in the number of nerves participating in the adrenergic ground plexus. The small intestine was an exception in that the final pattern of innervation in the wall was attained immediately after birth.

At the time of birth the short adrenergic neurons appeared in bundles encircling the vas deferens, and during the first two days post partum delicate fibres could be seen in the most peripheral parts of the smooth muscle layer 2—3 days later they formed a net work radiating through the wall of the organ. A dense ground plexus was first formed in the innermost parts of the proximal vas deferens, from where it then rapidly extended in a concentric manner to occupy the entire thickness of the musculature 8—10 days after birth. The density of the adrenergic innervation increased progressively during the following week and reached an almost adult pattern at 16 days. A similar course of development was observed in the distal vas deferens but the innervation appeared later than in the proximal portion.

The determination of endogenous NA contents of the heart and vas deferens as an indication of the density of the adrenergic innervation revealed rather parallel increase in both organs up to adult levels, which were reached 3—5 weeks after birth. A further increase in NA concentration of the vas deferens at older ages is often encountered both in rats (Sjöstrand and Swedin 1967 Livett 1968) and guinea pigs (Blakeley Dearnaley and Harrison 1970)

Thus, there was no overt difference in the rate of development of the terminal sympathetic innervation in organs supplied by short adrenergic neurons (accessory male genital organs) compared to the innervation of the submaxillary gland, heart and kidney which receive classical long adrenergic neurons. The intestine on the other hand, is innervated by long adrenergic neurons,

which reach an adult pattern of innervation in the effector organ at a distinct ly earlier stage than in the peripheral organs studied Nevertheless the intestinal adrenergic nerves are more affected by postnatally administered nerve growth factor-antiserum than the short adrenergic neurons (Hamberger *et al.* 1965 Zaimis, Berk and Callingham 1965 Iversen Glowinsky and Axelrod 1966) It is thus conceivable that other features than an early appearance distinguish the short adrenergic neurons from ordinary long ones with regard to their resistance to immunosympathectomy and that the chromaffin cells and the short adrenergic neurons possess some other common properties, not related to the time of appearance of CA in the cells.

B. Functional observations (Paper 2)

With the morphological observations as a basis it seemed to be of interest to establish also the functional development of the motor innervation of the rat vas deferens, as indicated by the appearance of a mechanical response of the longitudinal smooth muscle layer to transmural nerve stimulation.

The first responses to postganglionic nerve stimulation appeared in preparations from animals 3 days of age, whereas responses to exogenous NA and direct stimulation of the smooth muscle cells were obtained one day earlier Preparations of all ages (2—22 days) responded readily to low concentrations of NA (generally in the range of 0 2—1 0 μg/ml in the youngest preparations somewhat higher concentrations were usually needed) while ACh had to be added in much higher concentrations (10—100 μg/ml) to give direct responses of equal amplitude.

To describe the further development of the mechanical responses of the vas deferens more quantitatively these responses were related to the total length of the preparations (*cf* Fig 4 Chapter V) A gradual increase in the percentual shortening of the organs on nerve stimulation from less than 0 1 % at 3 days (20 imp/s) up to about 10 % at 15 days (10 imp/s) was observed.

The possibility to selectively stimulate the nervous structures within the organ by means of the used transmural field stimulation method was clearly demonstrated in the present study The threshold duration of the stimuli for evoking a *nerve induced* contraction of the organ was generally distinct between 0.05 and 0 06 ms with maximum amplitude obtained with pulse lengths between 0 1 and 0 6 ms. This nerve induced contraction which was heavily depressed or abolished by guanethidine was readily distinguishable from the direct smooth muscle response which after adrenergic receptor or neuron blockade, began to develop at about 2 ms duration with maximum between 5 and 10 ms.

The present study thus indicates a close correlation between the morphological development of the terminal adrenergic network of the rat vas deferens and the development of the mechanical responses of this organ to transmural nerve stimulation, both with regard to the starting point and the further progressive development during the first weeks of life. A similar course of morphological development, although with a few days delay was recently reported for the adrenergic innervation of the mouse vas deferens (Furness McLean and Burnstock 1970) Functional transmission, as indicated by registration of excitatory junction potentials, was however not established until 18 days post partum. This discrepancy will be commented upon in Chapter V

In Paper 2 the effect of adrenergic and cholinergic receptor blockers on the responses of the vasa deferentia of different ages to nerve stimulation was also studied These results will be summarized and discussed in Chapters V and VII

IV PHYSIOLOGICAL AND PHARMACOLOGICAL INFLUENCES ON THE NA CONTENT OF THE VAS DEFERENS

A. In vivo observations (Paper 3)

Earlier observations in the literature have indicated a rather low rate of NA turnover in the vas deferens (Austin Livett and Chubb 1967 a, b Laverty and Robertson 1967 Roth, Stjärne and Euler 1967) Thus, together with the findings that the NA *turnover in the vas deferens* as in other tissues is dependent on the nerve impulses flow (Alousi and Weiner 1966 Roth Stjärne and Euler 1966 Austin, Livett and Chubb 1967 a, Weiner and Rabadjija 1968 a, b) and that section of the hypogastric nerve does not influence the NA depletion from the genital organs after a low dose of reserpine *in vivo* (Sjöstrand and Swedin 1968 a) has indicated a low nerve impulse flow in the short adrenergic neurons *in vivo* The aim of the present study was to get some further information about the NA turnover in these neurons under *in vivo* conditions and also to study the influence of the normal nerve impulse flow on it.

Inhibition of NA synthesis by α MPT and subsequent estimation of the rate of NA depletion as a means of determination of the NA turnover offers the major advantage that the actual organ concentration of the drug can be measured together with the endogenous levels of NA. This is especially important in organs with relatively poor blood supply (*cf* Sjöstrand and Swedin 1968 a) Thus, in the present study the time course of NA depletion and organ contents of α-MPT after repeated doses of the methyl ester of α MPT (H44/68) were studied in the heart, innervated and decentralized submaxillary gland, seminal vesicle and vas deferens of the rat.

A definite difference in effect of α-MPT on the different peripheral NA stores was demonstrated. While the innervated hearts and submaxillary glands had lost 22 % and 36 % of their NA respectively in 10 h the short adrenergic neurons of the vas deferens and the seminal vesicle retained about normal NA levels even 16 h after repeated doses of α MPT despite very high organ concentrations of the drug and regardless of whether the adrenergic nerve supply via the hypogastric nerve was intact or not.

The most probable explanation for this lack of effect of inhibiting the NA synthesis seemed to be either an almost total absence of nerve impulse flow in the short adrenergic neurons *in vivo* and/or a very efficient reuptake and reuse of liberated NA with only negligible enzymatic destruction and losses of NA molecules to the blood.

B In vitro observations (Paper 4)

The most suitable way of testing these possibilities seemed to be to determine the effect of intense nerve stimulation *in vitro* on the NA content of the vas deferens in the presence of inhibitors of NA synthesis and reuptake in order to evaluate which of these processes is playing the major role in maintaining the transmitter homeostasis of the short adrenergic neurons

The nerve stimulation was applied as field stimulation for 30 s every min at 7 or 25 imp/s. α-MPT was again used as synthesis inhibitor and the used concentrations (4×10^{-4} M) resulted in organ contents of the drug comparable to those obtained in the *in vivo* study (Paper 3) which means a molar ratio of α-MPT to T of about 6 1 Among the various drugs known to inhibit NA uptake cocaine was chosen for the systematic study (*cf* Paper 4)

The results confirmed earlier observations (Roth Stjärne and Euler 1967 Blakeley Dearnaley and Harrison 1968 1970) that the endogenous stores of NA in the vas deferens are markedly resistant to even intense nerve stimulation for a long time. Furthermore it was shown that the presence of high concentrations of drugs known to inhibit uptake and synthesis of NA did not significantly increase the slight decrease in NA concentration of the vas deferens induced by the nerve stimulation *per se* DMI and PBA were also tested in concentrations up to levels where a further decrease in NA concentrations when compared to nerve stimulation as such was actually achieved. In the concentrations needed (10^{-4}—10^{-3} M) these drugs are certainly not working by inhibiting the NA uptake only (*cf* Paper 4) as indicated also by the significantly decreased NA levels observed even without nerve stimulation.

From these results it is obvious that no conclusion can be drawn about the nerve impulse flow in the short adrenergic neurons *in vivo* The most plausible explanation of the findings seems to be that these neurons normally are working with a very small pool of functional transmitter Since no pharmacological blockade of uptake or synthesis is 100 % effective, the residual function together with minute refilling from the large storage pools could be able to maintain the transmitter homeostasis in the vas deferens for a considerable time without measureable changes in the total endogenous content of NA. An additional explanation might be offered by the results of Paper 6, which are being presented and discussed in Chapters VI and VII

V BIPHASIC MECHANICAL RESPONSE OF THE ISOLATED VAS DEFERENS TO NERVE STIMULATION

(Paper 5)

In most studies using the isolated *in vitro* preparations of the vas deferens, the organ has been stimulated for shorter periods (2—10 s) every one or two min. During the preceding *in vitro* investigation (Paper 4) longer periods of stimulation, generally 30 s every min, were used. It was then constantly noted that the initial rapid nerve induced contraction ("twitch) of the organ after about 5 s was followed by a second slower phase of contraction (Fig 2) As it was shown that these two phases of contraction could be separately in fluenced by different drugs (*cf* Fig 2) a more systematic study was performed to elucidate the nature of the two phases (Paper 5 and unpublished observations)

It was found that both phases were present at all stimulation frequencies studied (1—50 imp/s) and that the separation into two phases was as a rule even more distinct in the guinea pig preparations, especially when stimulated via the hypogastric nerve.

Adrenergic α receptor blockers generally potentiated the twitch while the second phase of contraction was immediately and constantly suppressed or totally disappeared. β receptor blockers or atropine did not overtly change either of the phases while very low concentrations of PGE_1 markedly de creased or abolished the twitch" of the guinea pig preparation while leaving the second phase relatively uninfluenced at the concentrations used. Adrenergic neuron blockers (guanethidine, bretylium) profoundly depressed or completely abolished both phases. Reserpine pretreatment of the animals de creased the NA contents of the vasa deferentia, generally to below 1 % of the controls (see below) but nevertheless the organs responded with twitches" on nerve stimulation for up to 10 h. In no case was it possible to obtain a clearcut second phase of the reserpinized organs (Fig 3) To make sure that both phases really are nerve induced some experiments were performed on postganglionically denervated preparations, which did not respond to transmural stimulation of less than 10 ms duration.

From the present results it seems to be clear that the *second phase* of contraction is mediated by adrenergic nerves liberating NA on stimulation since it is classically sensitive to adrenergic receptor or neuron blockers, reserpine, and postganglionic denervation.

The nature of the initial *twitch*" needs some further consideration. Also

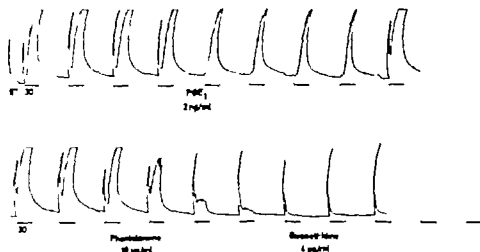
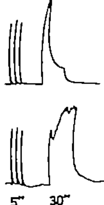


Fig. 2. Isolated guinea-pig vas deferens, stimulated via the hypogastric nerve as indicated by bars for 5 or 30 t 1 min interval with 25 V 8 imp/ 1.5 ms d ration. PGE phentolamine and guanethidine added as indicated at dot washing. Bath olume 50 ml. Lower curve obtained later in the same experiment (from Paper 5)

this seems to be induced by stimulation of adrenergic neurons, since it is abolished by adrenergic neuron blockade by guanethidine or bretylium, and by denervation. On the other hand, it is obviously not markedly influenced by reserpine pretreatment and not inhibited by moderate concentration of α- or β-blockers.

With regard to the Burn and Rand hypothesis (*cf* Introduction) and de spite its atropine and hyoscine resistance (Paper 5 Bell 1969) it seemed to be of importance to further investigate the possibility of an ACh mediated initiation of the contractile response of the vas deferens to transmural nerve stimulation. Neither of the other kinds of cholinergic receptor blockers, hexamethonium and d-tubocurarine did in concentrations up to 100 μg/ml overtly influence the "twitch" (see also Westwood and Whaler 1968) This is further known to be resistant to intense and prolonged nerve stimulation in presence of hemicholinium, an inhibitor of the ACh synthesis which heavily depresses the responses of the vas deferens to *preganglionic* nerve stimulation (Bentley and Sabine 1963) probably as a result of ACh depletion in the pre ganglionic nerve terminals. Taken together these findings seem to make ACh rather unlikely as the mediator of the twitch of the vas deferens in response to nerve stimulation.

Fig. 3 2 Isolated, field stimulated guinea-pig vas deferentia. Upper curve from an animal pretreated for 2 days with reserpine (3 6 and 6 mg/kg i.p. 48, 4 and 12 h before the expt) lower curve from an untreated control animal. Stimulated for 5 or 30 (10 times higher paper speed) with 20 V 1.5 ms duration t 4 imp/s (reserpinized) and 6 imp/ (control) Same amplification, bath volumes 5 ml.



The further pharmacological analysis was performed on vas deferens preparations from reserpine pretreated (*cf* paper 5 and 6) rats (n = 10) and guinea pigs (n = 8) It has already been shown (Bell 1969) that the remaining mechanical response to nerve stimulation of reserpinized preparations is resistant to hyoscine. It is further known that other adrenergically innervated preparations respond to nerve stimulation although their NA stores have been heavily depleted by reserpine (Sedvall and Thorson 1965 Andén and Henning 1966, Lee 1967) In the present study one vas deferens was taken for immediate NA determination while the other was mounted in the organ bath and stimulated. In the unstimulated control rat vasa deferentia almost unmeasurable quantities of NA remained (0 059 ± 0 039 μg/g, n = 9) In the stimulated preparations (10—25 imp/s, 5 or 30 s at 1 min interval for up to 6 h) the mean NA concentration after the experiments was significantly higher (0.468 ± 0.134 μg/g, n = 9 0 01 > p > 0 001) indicating that some NA synthesis could be induced by the nerve stimulation despite heavy reserpine pretreatment (*cf* Alousi and Weiner 1966, Roth and Stone 1968 Häggendal and Malmfors 1969) Furthermore, it is obvious that if NA is the transmitter of the twitch the functional transmitter pool necessary for evoking this initial nerve induced contraction, is very small compared to the normal storage pool in the short adrenergic neurons.

After the establishment of the marked endogenous inhibition of the mechanical responses of the nerve stimulated vas deferens (Paper 6) it became evident that no decrease of the mechanical response of the preparations could be correctly interpreted unless this autoinhibitory mechanism was taken into account. Some additional experiments were therefore performed on vas deferens

preparations from reserpine pretreated guinea pigs, where the autoinhibition had been reduced by *in vitro* blockade of the PG synthesis with ETA (20 μg/ml, *cf* Paper 6) In the presence of α-MPT (10^{-4} M) intense nerve stimulation (50 imp/s for 30 s every min) resulted in a definite decrease in the mechanical responses of th preparations. With current methods it seems however to be impossible to decide whether this decrease really depends on exhaustion of the small functional pool of NA, or to some other factors.

Perhaps the final answer to the question about the initiation of the "twitch" and the relative role of cholinergic and adrenergic components in the mechani cal response of the nerve stimulated vas deferens is given by the results of the young rat preparations, presented in Paper 2. Here it was shown that adrenergic α-blockers in low concentrations definitely abolished the 'twitch of preparations up to about 10 days of age whereafter the effect was changed to none or as generally in adult preparations a potentiation of the responses (Fig 4) Atropine (0.5—2.0 μg/ml) usually caused some reduction (10—40 %) of the contraction amplitude of preparations up to about 15 days of age, while it at older stages was without effect.

It was concluded that the major part of the motor innervation of the rat vas deferens is classically adrenergic and responsible for both the twitch" and the second phase of contraction. A probable explanation to the biphasic pattern of the nerve-induced mechanical response of the vas deferens will be presented in Chapter VII

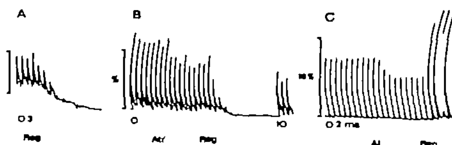


Fig 4 Isolated field stimulated vas deferens preparations from rats of different ages. Duration (ms) of the stimuli indicated in the figure. The amplitude of th contractions are compared (%) to the total length of th preparations. A *5 days* length 17 mm; 20 V 25 imp/s, 0.3 ms. Reg phentolamine 2 μg/ml. B *8 day* length 23 mm 20 V 10 imp/s, 0 4 or 10 ms duration. Atr atropine 2 μg/ml. Reg phentolamine 2 μg/ml. C *15 days* length 18 mm 20 V 10 imp/s, 0.. ms. Atr: tropine 1 μg/ml. Reg phentolamine 1 μg/ml. (from Paper 2)

Furthermore, the atropine sensitivity of a part of the mechanical response in preparations from rats younger than about 15 days, seems to indicate that ACh actually is liberated from some source within the organ on transmural stimulation. The released amounts are, however too low to evoke a registrable mechanical response of the organs after adrenergic receptor blockade and the ACh is thus most probably working as a potentiating agent for the released NA (Sjöstrand 1961 Sjöstrand and Swedin 1968 b) This supports the existence also in the rat vas deferens of a minor probably independent cholinergic nerve supply as has been suggested for the guinea pig (for ref see Paper 2) and mouse vas deferens (*cf* Burnstock 1970)

In Paper 2 it was suggested that the postnatal change in effects of the α-blockers and atropine could be due to altered accessibility of the junctional receptors during the first weeks of life. Th inability to evoke excitatory junction potentials from the mouse vas deferens until about the 18th day post partum when the adrenergic nerves appear fully developed histochemically (Furness, McLean and Burnstock 1970) seems also to justify the interpretation, that the neuromuscular junction might be immature and/or less intimate even a considerable time after that a functional neurotransmission can be established (*cf* Paper 2 Chapter II)

VI ENDOGENOUS INHIBITION OF THE NERVE-INDUCED MECHANICAL RESPONSE OF THE VAS DEFERENS

(Paper 6)

During the course of the *in vitro* studies described in Papers 4 and 5 it was observed that repeated periods of prolonged nerve stimulation (30 s every min) of the isolated rat or guinea pig vas deferens tended to depress especially the "twitch" of subsequent contractions of the organ. At higher stimulation frequencies and especially in the rat preparation this depression was preceded by increased height of contraction (Fig 5) The nature and degree of inhibition was dependent on species, temperature and frequency of stimulation.

The following findings led to the conclusion that the inhibition was induced by some substance released together with the neurotransmitter upon nerve stimulation 1 The inhibition was immediately abolished on washing of the organ. 2. It appeared faster and was more complete in an organ bath of small volume than in one of a greater 3 It was possible to transfer the substance with its inhibiting properties from one bath to influence an organ in another

The inhibitory agent is probably working on a prejunctional level since tests with exogenous NA or ACh revealed unaltered responses of the preparations after periods with 30 s stimulation.

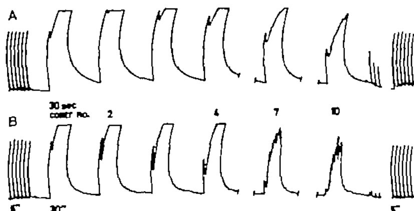


Fig. 5 Isolated field stimulated vas deferens preparations from rat (A) and guinea-pig (B) in 5 ml baths. 20 V 1.5 ms duration, 5 imp/s, for 5 or 30 (10 times higher paper speed) at 1 min intervals. At dots washing. (from Paper 6)

The following results lended support to the hypothesis that the inhibitory agent could be related to PG 1 The inhibitory agent influences both phases of contraction, the second phase however to less extent, as do exogenously administered PGE_1 and PGE_2 2 The described species difference with regard to the autoinhibition, the guinea pig vas deferens being the more susceptible, also supports the concept of a PG mechanism since the guinea pig preparations are far more sensitive also to exogenous PG (Hedqvist and Euler 1971) 3 The ganglionic relay existent in the most peripheral part of the hypogastric nerve was found to be extremely sensitive to the inhibiting substance and the same was found for exogenous PG (0 1—0 4 ng/ml) 4 Incubation of the vas deferens with an inhibitor of PG synthesis (ETA) led to a partial or total abolishment of the endogenous inhibition 5 Release upon continuous nerve stimulation from the vas deferens of PG-like material mainly resembling PGE_2 was established.

From the present results the conclusion can be drawn that the nerve induced mechanical response of the isolated vas deferens is under a dual influence from excitatory and inhibitory agents. It seems highly probable that the endogenous inhibition is brought about by release of PG which is known to restrict the transmitter release from sympathetic nerves (*cf* Hedqvist 1970 Hedqvist and Wennmalm 1971) Recently it has furthermore been shown that the transmitter secretion from the adrenergic neurons of the rabbit heart is limited by an endogenous PG-mediated mechanism (*cf* Wennmalm 1971 b)

The existence of such an autoinhibitory mechanism also in the vas deferens might perhaps offer an explanation to some of the confusing results obtained in studies on this organ In fact, the described autoinhibition has been repeatedly observed especially in reserpinized preparations (Huković 1961 and others) where the progressive decrease in mechanical response has been interpreted as due to exhaustion of the severely depleted NA stores (*cf* Paper 6) Also in normal preparations it has been observed but neither discussed nor explained (Weiner and Rabadjija 1968 b Palaić and Panisset 1969 Blakeley Dearnaley and Harrison 1970)

An effective endogenous restriction of the NA release on nerve stimulation seems to be the most attractive explanation of the resistance of the NA stores of the vas deferens to even intense nerve stimulation (*cf* Paper 4 Chapter IV) Furthermore the nerve induced mechanical response levels off very soon after initiation of a continuous nerve stimulation of the vas deferens (Weiner and Rabadjija 1968 b Palaić and Panisset 1969) Even if the amplitude of the contractile response presumably gives only an approximate reflection of the amount of transmitter released this decay might indicate that the release of NA rapidly is restricted to very low amounts on continuous nerve stimulation.

This would then explain the finding of Weiner and Rabadjija (1968b) that intermittent nerve stimulation induces a greater increase of NA synthesis in the vas deferens than does continuous stimulation

The endogenous inhibitory mechanism might also be involved in some other controversial features regarding the neurotransmission of the vas deferens. Thus it has been reported that exogenous NA sometimes exerts an inhibitory action on the nerve induced contractions of the vas deferens (Holman and Jowett 1964 Van Orden Bensch and Glarman 1967 Hotta 1969 Ambache and Zar 1970) This effect has been explained as due to stimulation of adrenergic β-receptors (Holman and Jowett 1964 Large 1965 Takagi and Takayanagi 1965) or to a desensitizing action of NA on adrenergic α-receptors (Hotta 1969) The last explanation seems on electrophysiological grounds to be somewhat doubtful (Sjöstrand 1971) An additional alternative, based on the present results, might be that the exogenous NA, although present in subthreshold concentrations for a contractile response of the organ induces release of PG (*cf* Ferreira and Vane 1967 Gilmore Vane and Wyllie 1968) which restricts the subsequent release of NA on nerve stimulation.

It has been shown (Davies, Horton and Withrington 1967 1968 Ferreira and Vane 1967) that α-blockers diminish the nerve induced release of PG from the perfused dog spleen, and suggested (Hedqvist 1969 1970 Wennmalm 1971 a, b) that part of the increase in NA overflow caused by α-blockers in perfused, nerve stimulated organs could be due to such an inhibition of endogenous PG release. By analogy a subtotal blockade of the α-receptors in the vas deferens (*cf* Paper 2) might perhaps lead to an inhibition of the release of PG without preventing the initiation of a contractile response of the effector organ, which could in part explain the potentiation of the mechanical response of the vas deferens induced by α-blockers in adult preparations (*cf* Papers 2 and 5 Fig 2 and 4) Direct effects of the α-blockers, especially in higher concentrations, on the nerve terminals or the smooth muscle cells cannot, however be excluded.

The aim of the present investigation was to collect some further information about certain aspects of the motor innervation of the rat and guinea pig vas deferens. The study has included observations on the morphological and functional development of this innervation postnatally in vitro observation of a characteristic, biphasic pattern of the mechanical response of the vas deferens to nerve stimulation including pharmacological analysis of the two phases and the participation of separate adrenergic and cholinergic components of the nerve supply of the rat vas deferens in vivo and in vitro studies on the transmitter homeostasis of the adrenergic neurons of the rat vas deferens and observation of an endogenous auto-inhibition of the mechanical response of the vas deferens to nerve stimulation

The intention of this chapter is to provide a reasonable model of the adrenergic neurotransmission of the vas deferens based upon the present results in view of earlier knowledge of it.

In most mammals the vas deferens seems to be the most densely innervated smooth muscle organ (*cf* Sjöstrand 1965) The ultrastructural picture of the neuromuscular junctions of this organ reveals some characteristic features which clearly distinguishes it from that obtained in *e g* the intestinal or vascular smooth muscle (*cf* Burnstock 1970) Thus all the muscle cells of the rat and mouse vas deferens (Richardson 1962, Lane and Rhodin 1964) seem to receive individual innervation, whereas in the guinea pig organ less than half of the cells have close neuromuscular contact (Merrillees, Burnstock and Holman 1963 Merillees 1968) The terminal nerve fibres usually run in shallow depressions of the muscle membrane or may even be enclosed within the smooth muscle cell (*cf* Furness and Iwayama 1971) Furthermore, the proportion of these close neuromuscular junctions (*i.e* about 200 Å in the rat and guinea pig 70—80 Å in the mouse vas deferens) is high when compared to the intestinal and vascular musculature, where the junctional gap rarely is less than 800 Å (*cf* Burnstock 1970)

The electrophysiological implications of these morphological observations have recently been reviewed by Holman (1970) There seems to be certain discrepancies in the electrophysiological responses of the rat mouse and guinea pig vas deferens these discrepancies might in part be based on the morphological differences between the species (*cf* Furness and Burnstock 1969) Nevertheless, the efficiency of the transmission processes in the vas deferens, as indicated by the electrophysiological characteristics, seems to be

[illegible] neurotransmission mechanisms of the motor inner[illegible] guinea pig vas deferens, the main results are the follow[illegible]

1. The [illegible] development of the mechanical responses of the rat vas [illegible] transmural nerve stimulation appears to proceed rather parallel [illegible] morphological development of the short adrenergic neurons inner[illegible] [illegible] organs as indicated by the histofluorescence picture of [illegible]. The first responses to nerve stimulation are obtained in preparations [illegible] 3 days of age, and an adult morphological and functional stage is [illegible] within the first 3—4 weeks of life.

   For this study a superfusion bath was constructed, which, together with the electronic transducer and amplifier used, permits registration of mechanical responses with an amplitude of less than 10 [illegible]

2. The endogenous NA stores of the rat vas deferens are markedly resistant to both blockade of NA synthesis *in vivo* and blockade of synthesis and uptake of NA in combination with nerve stimulation *in vitro*.

3. The nerve-induced mechanical response of the rat and guinea pig vas deferens is characteristically divided in an initial, rapid phase ("twitch") and a subsequent second slower phase of contraction when the usual 2—5 s of stimulation every min is extended to 30 s. The two phases can be separately influenced by certain anti adrenergic drugs. The most probable explanation of this biphasic pattern of nerve-induced responses seems to be that the "twitch" is induced by the initial release of NA, instantaneously reaching a high local concentration within the close neuromuscular gap existing in this organ, while the second phase is mediated by stimulation of recept[illegible] located outside the neuromuscular junction.

4. The *in vitro* effects of adrenergic [illegible]-blockers and atropine are changed during the postnatal development of the motor innervation of the rat vas deferens. Up to 10—15 days post partum the α blockers cause a total inhibition and atropine a partial reduction of the mechanical responses to transmural nerve stimulation. Later on the [illegible] the adult pattern, generally a potentiation of the [illegible] and no effect of atropine. It is concluded that the [illegible]

VIII SUMMARY

In the present study on neurotransmission mechanisms of the motor innervation of the rat and guinea pig vas deferens, the main results are the following

1 The postnatal development of the mechanical responses of the rat vas deferens to transmural nerve stimulation appears to proceed rather parallel to the morphological development of the short adrenergic neurons innervating the pelvic organs, as indicated by the histofluorescence picture of these. The first responses to nerve stimulation are obtained in preparations from rats 3 days of age and an adult morphological and functional stage is reached within the first 3—4 weeks of life.

   For this study a superfusion bath was constructed, which together with the electronic transducer and amplifier used, permits registration of mechanical responses with an amplitude of less than 10 μ

2. The endogenous NA stores of the rat vas deferens are markedly resistant to both blockade of NA synthesis *in vivo* and blockade of synthesis and uptake of NA in combination with nerve stimulation *in vitro*

3 The nerve-induced mechanical response of the rat and guinea pig vas deferens is characteristically divided in an initial, rapid phase ("twitch") and a subsequent second, slower phase of contraction when the usual 2—5 s of stimulation every min is extended to 30 s. The two phases can be separately influenced by certain anti adrenergic drugs. The most probable explanation of this biphasic pattern of nerve induced responses seems to be that the twitch is induced by the initial release of NA, instantaneously reaching a high local concentration within the close neuromuscular gap existing in this organ, while the second phase is mediated by stimulation of receptors located outside the neuromuscular junction

4 The *in vitro* effects of adrenergic α-blockers and atropine are changed during the postnatal development of the motor innervation of the rat vas deferens. Up to 10—15 days post partum the α blockers cause a total inhibition and atropine a partial reduction of the mechanical responses to transmural nerve stimulation. Later on the effects are changed into the adult pattern generally a potentiation of the responses to α-blockers and no effect of atropine It is concluded that the major part of the motor

IX. ACKNOWLEDGEMENTS

I wish to express my deep gratitude to my teacher Dr Nils O Sjöstrand, who introduced me into the tricky field of vas deferens physiology. His generous supply with advice and criticism, his unconventional approach to scientific problems together with a really profound knowledge of physiology has been of invaluable importance to me, as well as the warm companionship he has offered me during quite a number of nights in front of the isolated organ baths.

I am further much indebted to Professor U S v Euler, director of the department, for his unfailing interest, valuable suggestions and support in different ways.

My warm thanks are also due to Drs. Christer Owman and Nils-Otto Sjöberg with their skilful technical staff at the Department of Histology, University of Lund, for their kind hospitality, patience before and stimulating co-operation after I had learned a bit about fluorescence microscopy.

The excellent technical assistance of Mrs. Kerstin Westling, Birgitta Tengstrand and Annika Rosén, is gratefully acknowledged.

I am also very grateful to Mrs Vera Rydberg, who typed all the manuscripts, as well as to all the members of the staff of the Department of Physiology for their kind help and interest in my work.

Finally I want to thank my parents for kind support and hospitality during some wonderful summermonths in the archipelago of Stockholm, where this thesis was ultimately formulated.

The work was supported by grants from the Medical Faculty, Karolinska Institutet, Stiftelsen Lars Hiertas Minne, Svenska Sällskapet för Medicinsk Forskning and Swedish Medical Research Council (project no. B71 14X-3186-01).

IX. ACKNOWLEDGEMENTS

I wish to express my deep gratitude to my teacher Dr Nils O Sjöstrand, who introduced me into the tricky field of vas deferens physiology His generous supply with advice and criticism, his unconventional approach to scientific problems together with a really profound knowledge of physiology has been of invaluable importance to me as well as the warm companionship he has offered me during quite a number of nights in front of the isolated organ baths.

I am further much indebted to Professor U S v Euler director of the department, for his unfailing interest, valuable suggestions and support in different ways.

My warm thanks are also due to Drs. Christer Owman and Nils-Otto Sjöberg with their skilful technical staff at the Department of Histology University of Lund, for their kind hospitality patience before and stimulating co-operation after I had learned a bit about fluorescence microscopy

The excellent technical assistance of Mrs. Kerstin Westling, Birgitta Tengstrand and Annika Rosén, is gratefully acknowledged.

I am also very grateful to Mrs Vera Rydberg, who typed all the manuscripts as well as to all the members of the staff of the Department of Physiology for their kind help and interest in my work.

Finally I want to thank my parents for kind support and hospitality during some wonderful summermonths in the archipelago of Stockholm, where this thesis was ultimately formulated.

The work was supported by grants from the Medical Faculty Karolinska Institutet, Stiftelsen Lars Hiertas Minne, Svenska Sällskapet för Medicinsk Forskning and Swedish Medical Research Council (project no B71 14X 3186-01)

Burnstock, G Structure of smooth muscle and its innervation. In "Smooth Muscle Eds. E. Bülbring, A. Brading, A. Jones and T Tomita. Edw Arnolds Publ. London 1970 1—69

Burnstock, G and M. E. Holman, A tonomic nerv -smooth muscle transmission. N ture (*Lond*) 1960. *187* 951—952.

Champlain, J de, T Malmfors, L. Olsson and Ch. Sachs, Ontogenesis of peripheral adrenergic neurons in the rat pre and postnatal observ tions. *Act physiol. scand* 1970. *80* 276—288

Chang C C., A sensitiv method for spectrophotofluorometric assay of catecholamines. *Int. J Neur pharmacol.* 1964 *3* 643—649

Chang, C. C. and J C. Chang, A change in the subcellular distribution of noradrenaline in the rat isolated vas deferens effected by nerve stimulation. *Brit J Pharmacol.* 1965 *25* 758—762.

Davies B. N E. W Horton and P G Withrington, The occurrence of prostaglandin E in splenl enous blood of the dog following splenic nerv stimulation. *J Physiol.* (*Lond*) 1967 *188* 38—39P

Davies, B. N E. W Horton and P G Withrington, Th occurrence f prostaglandin E_2 in splenic venous blood of the dog following splenic nerv timulation. *Brit J Pharmacol.* 1968. *32* 127—135.

Euler U S. v Release and uptake of noradrenaline in adrenergic nerve granules. *Act physiol. c d* 1966 67 430—440

Euler U S v and F Lishajko A specific kind of noradrenaline granules in the vesicula gland and the as deferens of the bull. *Lif Sci* 1966 *5* 687—691

Falck, B., Ch. Owman and N O Sjöstrand, Peripherally located adrenergic neurons innervating vas deferens and the seminal vesicle of the guinea-pig *Experientia* (*Basel*) 1965 *21* 98—100

Ferreira, S. H. and J R. Vane, Prostaglandins Their disappearance from and release into the circulation. *Nature* (*Lond*) 1967 *216* 868—873

Ferry C. B., The innervation of the as deferens of th guinea-pig. *J Physiol.* (*Lond*) 1963 *166* 16P

Ferry C. B The innervation of the as deferens of th guinea-pig *J Physiol.* (*Lond*) 1967 *19?* 463—478.

Furness, J B. and G. Burnstock, A comparativ study of spike potentials in response to nerv timulation in the vas deferens of the mouse t and guinea-pig *Comp. Biochem. Physiol* 1969 *31* 337—345

Furness, J B. and T Iwayama, Terminal axons ensheathed in smooth muscle cells of the vas deferens. *Z Z llfor h* 1971 *113* 259— 70

Furness, J B J R. McLean and G Burnstock, Distribution of adrenergic nerves and changes in neuromuscular transmission in the mouse vas deferens during postnatal development. *Develop Biol.* 1970. *1* 491—505.

Gershon, M. D The identification of neurotransmitters t smooth muscle. In "Smooth Muscle Eds. E. Bülbring, A. Brading, A. Jones and T Tomita. Edw Arnold Publ. London 1970. 496—524

Gilmore N J R. Vane and J H Wyllie Prostaglandins released by the spleen. N tu (*Lond*) 1968 *218* 1135—1140

Häggendal, J and T Malmfors, The effect of nerve stimulation on catecholamines taken up in adrenergic nerves after reserpine pretreatment. *Act physiol scand* 1969 *75* 33—38.

Burnstock, G. Structur of smooth muscle and its innervation. In "Smooth Muscle" Eds. E. Bülbring, A. Brading, A. Jones and T. Tomita. Edw. Arnolds Publ. London 1970. 1—69

Burnstock, G. and M. E. Holman, Autonomic nerv -smooth muscle transmission. *Nature (Lond.)* 1960 *187* 951—952

Champlain, J. de, T. Malmfors, L. Olsson and Ch. Sachs, Ontogenesis of peripheral adrenergic neurons in the rat pr and postnatal observations. *Acta physiol. scand* 1970 *80* 276—288

Chang, C. C., A sensitive method for spectrophotofluorometric assay f catecholamines. *Int J Neu pharmacol.* 1964 *3* 643—649

Chang, C. C. and J. C. Chang, A change in the subcellular distribution of noradrenaline in the rat isolated vas deferens effected by nerve stimulation. *Brit J Pharmacol.* 1965. *25* 758—762.

Davies, B. N. E. W. Horton and P. G. Withrington, The occurrence of prostaglandin E₂ in splenic venous blood of the dog following splenic nerve stimulation. *J Physiol. (Lond.)* 1967 *188* 38—39P

Davies, B. N. E. W. Horton and P. G. Withrington, The occurrence of prostaglandin E₂ in splenic venous blood of the dog following splenic nerv timulation. *Brit. J Pharmacol* 1968. *32* 127—135

Euler U. S. v. Release and uptake of norad enaline in adrenergic nerve granules. *A ta physiol. scand* 1966 *67* 430—440.

Euler U. S. and F. Lishajko A specific kind of noradrenaline granules in the esicular gland and the vas deferens of the bull. *Lif Sci* 1966 *5* 687—691

Falck. B., Ch. Owman and N. O. Sjöstrand, Peripherally located adrenergic neurons innervating vas deferens and the seminal esicle of the guinea-pig. *Experientia (Basel)* 1965 *21* 98—100

Ferreira, S. H. and J. R. Vane, Prostaglandins Their disappearance from and release into the circulation. *Nat (Lond.)* 1967 *216* 868—873

Ferry C. B. The innervation of the as deferens of the guinea-pig. *J Physiol. (Lond.)* 1963 *166* 16P

Ferry C. B., The innervation of the vas deferens of the guinea-pig. *J Physiol. (Lond.)* 1967 *192* 463—478

Furness, J. B. and G. Burnstock, A comparativ study of spike potentials in response to nerv stimulation in the vas deferens of the mouse, t and guinea-pig. *C mp Biochem Physiol* 1969 *31* 337—345

Furness J. B. and T. Iwayama, Terminal axons ensheathed in smooth muscle cells of the vas deferens. *Z Zellforsch* 1971 *113* 259—270.

Furness, J. B., J. R. McLean and G. Burnstock, Distribution of adrenergic nerves and changes in neuromuscular transmission in the mouse vas deferens during postnatal development. *Develop Biol* 1970. *21* 491—505.

Gershon, M. D. The identification of neurotransmitters to smooth muscle. In "Smooth Muscle Eds. E. Bulbring, A. Brading, A. Jones and T. Tomita. Edw. Arnold Publ. London 1970 496—5 4

Gilmore, N. J. R. Vane and J. H. Wyllie, Prostaglandins released by the spleen. *Nat (Lond.)* 1968. *218* 1135—1140

Häggendal, J. and T. Malmfors, The effect of nerve stimulation on catecholamines taken up in adrenergic nerves after reserpine pretreatment. *Acta physiol. scand* 1969 *75* 33—38.

Weiner N and M Rabadjija, The regulation of norepinephrine synthesis. Effect f puromycin on the accelerated synthesis of norepinephrine associ ted with nerve stimulation. *J Pharmacol. exp Ther* 1968 b *164* 103—114

Wennmalm, A., Quantitative evaluation of release and reuptake of adrenergic transmitter in the rabbit heart. *Acta physiol. scand* 1971 a. *82* 532—538.

Wennmalm, A., Studies on mechanisms controlling the secretion of neurotransmitters in the rabbit heart. *Act physiol. c d* 1971 b. *82* Suppl. 365

Westwood, D A. and B. G. Whaler Postganglionic paralysis of the guinea-pig hypogastric nerve- as deferens preparation by *clostridium botulin m* D toxin *Brit J Pharmacol.* 1968 *33* 21—31

Zaimis, E., L. Berk and B. A. Callingham, Morphological biochemical and functional changes in the sympathetic nervous system of rats treated with nerve growth fact antiserum. *Natu (Lond)* 1965 *206* 1220—1222

Weiner N and M. Rabadjija, The regulation of norepinephrine synthesis. Effect of puromycin on the accelerated synthesis of norepinephrine associated with nerve stimulation. *J Pharmac. exp Ther* 1968 b. *164* 103—114

Wennmalm, A., Quantitative evaluation of release and reuptake of adrenergic transmitter in the rabbit heart. *Act physiol. scand* 1971 a. *8* 532—538.

Wennmalm, A., Studies on mechanisms controlling the secretion of neurotransmitters in the rabbit heart. *Acta physiol. scand* 1971 b. *82* Suppl. 365

Westwood, D A and B. C. Whale Postganglionic paralysis of the guinea-pig hypogastric nerve-vas deferens preparation by Clostridium botulinum D toxin. *Brit J Pharmacol.* 1968 *33* 21—31

Zaimis, E., L. Berk and B. A. Callingham Morphological biochemical and functional changes in the sympathetic nervous system of rats treated with nerve growth factor antiserum. *Nature (Lond.)* 1965 *206* 1220—1222.

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 370

From the Department of Pharmacology University of Lund Sweden

THYROID HORMONE SECRETION

Its Regulation By Intrathyroidal Amines

by

ARNE MELANDER

LUND 1971

Printed in Sweden
Studentlitteratur
Lund 1971

This dissertation summarizes the following papers which will be referred to by the figures 1 - 12 given below

1 C RERUP and A MELANDER On the bioassay of thyrotrophin in plasma Acta endocr 1965 50 177 - 194

2 A MELANDER and C RERUP Studies on the thyroid activity in the mouse 1 The effects of thyroxine corticosteroids hypophysectomy and changes in environmental temperature Acta endocr 1968 58 209 - 214

3 A MELANDER Studies on the thyroid activity in the mouse 2 The effects of stress and other changes in corticotrophin secretion Acta endocr 1970 64 569 - 576

4 A MELANDER Amines and mouse thyroid activity: A thyroid--stimulating effect of 5-hydroxytryptamine and dopamine Acta endocr 1969 62 565 - 576

5 A MELANDER Amines and mouse thyroid activity Release of thyroid hormone by catecholamines and indoleamines and its inhibition by adrenergic blocking drugs Acta endocr 1970 65 371 - 384

6 L.E ERICSON A MELANDER CH OWMAN and F SUNDLER Endocytosis of thyroglobulin and release of thyroid hormone by catecholamines and 5 hydroxytryptamine Endocrinology 1970 87 915 923

7 A MELANDER Interactions of adrenergic blocking drugs with the in vivo release of thyroid hormone induced by thyrotrophin and the long acting thyroid stimulator Acta endocr 1971 66 151 161

8 A MELANDER and F SUNDLER Interactions between catecholamines 5-hydroxytryptamine and TSH on the secretion of thyroid hormone Endocrinology 1972 90 (in press)

9 A MELANDER CH OWMAN and F SUNDLER TSH induced appearance and stimulation of amine-containing mast cells in the mouse thyroid. Endocrinology 1971 89 528 - 533

10 L E ERICSON R. HÅKANSON A MELANDER CH OWMAN and F SUNDLER TSH induced release of 5-HT and histamine from thyroid mast cells in the rat Endocrinology 1972 90 (in press)

11 A MELANDER and F SUNDLER Significance of thyroid mast cells in thyroid hormone secretion Endocrinology 1972 90 (in press)

12 A MELANDER E NILSSON and F SUNDLER Sympathetic activation of thyroid hormone secretion in mice Endocrinology 1972 90 (in press)

CONTENTS

I INTRODUCTION

The mammalian thyroid gland is derived from two different embryonic structures and accordingly comprises two endocrine cell systems with different functions (Arey 1966 Boyd 1964 Pitt-Rivers and Trotter 1964 Copp 1969) The major part arises from the fore-gut (Arey 1966 Boyd 1964) and some of its features remind of this origin Its functional unit is a follicle with a lumen a parallel to the gastrointestinal tract (cf Seljelid 1966) and like gastrointestinal cells the thyroid follicle cells secrete products in two directions into the lumen and into vessels on the contraluminal side of the cells (Stein and Gross 1964 Wollman et al 1964 Wetzel et al 1965 Ekholm and Smeds 1966 Seljelid 1966 see below) The follicle cells and their hormone(s) - "thyroid hormone" - participate in the regulation of metabolism

The second endocrine cell system in the mammalian thyroid consists of the parafollicular cells (C cells) which originate from the last branchial pouches They occur in the ultimobranchial bodies during early embryonic life and remain there throughout life in non mammals (Copp 1969 1970) Several organs of branchial genesis - parathyroids carotid and aortic bodies and gills are involved in the regulation of ion - acid - base balance (cf Copp 1970) and this seems to be true for the C cells as well since they produce a polypeptide hormone calcitonin which lowers the plasma level of calcium ions The secretion of this hormone appears to be regulated by the blood level of Ca^{++} (Copp 1969 1970)

The secretion of thyroid hormone from the follicle cells is generally considered to take place as follows Thyroid hormone is synthetized as part of a large glycoprotein thyroglobulin which is stored in the follicular lumen as the "colloid" By endocytosis thyroglobulin re-enters the follicle cells It is broken down by the action of lysosomal hydrolytic enzymes and the thyroid hormone thus split off is transferred to the blood (Stein and Gross 1964 Wollman et al 1964 Wetzel et al 1965 Ekholm and Smeds 1966 Seljelid 1966 Kosanović et al 1968) So far no other mechanism for thyroid hormone secretion has been demonstrated Both synthesis and secretion of thyroid hormone is stimulated by the pituitary hormone thyrotrophin (TSH) (Werner 1963 Purves 1964) The secretion of TSH in turn is inhibited by thyroid hormone and stimulated by the hypothalamic TSH releasing hormone (TRH formerly TRF) whereby the secretion of

thyroid hormone is adapted to the body needs (v Euler and Holmgren 1956 a b Reichlin 1963 Purves 1964 Bowers et al 1967 1968 Guillemin 1967 Schally et al 1967 1969)

The importance of stimuli other than TSH in thyroid hormone secretion is less well established By intrathyroidal sympathetic and vagal fibres both noradrenaline (NA) and acetylcholine can reach the follicles (cf Söderberg 1958 1959 Ekholm and Zelander 1960 Falck et al 1964 a b Larson Owman and Sundler 1966) C cells and mast cells within the thyroid constitute other sources of highly active amines such as 5-hydroxytryptamine (5 HT) dopamine (DA) and histamine (cf Falck et al 1964 a b Larson Owman and Sundler 1966 Clayton and Szego 1967 Clayton and Masuoka 1968 Falck and Owman 1968) This dissertation summarizes some studies on the secretion of thyroid hormone with special regard to the influence of intrathyroidal arylethylamines It is shown that secretion of thyroid hormone can be evoked by both catecholamines and 5-HT that stimulation of the cervical sympathetic nerves can induce secretion of thyroid hormone through a direct action on the follicle cells by NA released from intrathyroidal adrenergic terminals and that thyroid mast cells can facilitate thyroid hormone secretion by releasing 5-HT and histamine upon stimulation by TSH

II MATERIAL AND METHODS

Thyroid hormone secretion was investigated by measurements of blood ^{131}I and plasma protein-bound ^{131}I (PB ^{131}I) levels and by light- and electron-microscopic studies of the endocytosis of thyroglobulin Mast cells and sympathetic nerve terminals were studied by electron- light and fluorescence-microscopic investigations and determination of intrathyroidal amines was made by fluorometry Detailed methodological descriptions are given in the separate reports (papers 1 – 12) and a summary follows below

Animals A total of 10 900 female NMRI mice (Lab Animal Breeding and Research Centre Ry Denmark) and 225 male and female Sprague-Dawley rats (Anticimex Stockholm Sweden) were used. Their average weights at the time of experiments were 20 g and 50 100 g respectively They were given tap water and a standard pellet diet (SAN Bolagen Malmö Sweden or EWOS Södertälje Sweden) unless otherwise specified. Blood samples were taken by puncture of

the orbital venous plexus and plasma was prepared by centrifuging blood in a Beckman-Spinco Microfuge (papers 1 and 2)

Drugs and hormone preparations were administered orally (in the drinking water or in the food) subcutaneously (dorsal neck region) intraperitoneally (central part of the abdominal cavity) or intravenously (tail vein) Hypophysectomy was performed by the transauricular technique (Falconi and Rossi 1964 papers 2 4 5 and 6) and adrenalectomy by the lumbar approach (paper 3) Both surgical isolation of the cervical sympathetic trunk and cervical sympathectomy were made under stereo-microscopic observation (paper 12) Anaesthesia was induced either by intraperitoneal injections of pentobarbital or by inhalation of diethyl ether Animals were sacrificed by dislocation of the neck or by a blow on the head

Blood ^{131}I and PB^{131}I measurements Na ^{131}I (*Atomenergi* Studsvik Sweden) was injected intraperitoneally after the animals had been kept for 7 - 14 days on a diet with a low or a moderate iodine content (cf paper 1) Blood ^{131}I activity was measured in a gas flow counter with end-window (Nuclear-Chicago) and PB ^{131}I activity was determined in an auto-gamma spectrometer (Packard) after precipitation and two washings with cold 10% trichloroacetic acid (papers 2 - 5)

Light microscopy This was used for the demonstration of colloid droplet formation in thyroid follicle cells and for the detection and location of mast cells Colloid droplet formation After sacrifice the thyroid with adjacent tissue was taken out fixed for 18 h at 4°C in Bouin s solution dehydrated embedded in paraffin sectioned at 6 μ and stained by the periodic acid - Schiff (PAS) technique (papers 6 8 11 and 12) In general the number of PAS-positive cellular inclusions was obtained by counting 25 follicles (from the centre to the periphery in the widest area of the thyroid section) in each of the two lobes <u>Mast cells:</u> Sections from freeze-dried formaldehyde-treated material or from glutaraldehyde-fixed tissue (see below) were stained in an ethanol solution of toluidine blue (1%) for the light microscopic demonstration of mast cells (Romeis 1948) (papers 9 - 11)

Electron microscopy Anaesthetized animals were perfused via the ascending aorta with about 50 ml of 3% glutaraldehyde in 0 075 M sodium cacodylate pH 7 2 Thyroid specimens were excised and kept in the glutaraldehyde fixative for 1 2 h They were postfixed for 2 h in 1% osmium tetroxide in Veronal acetate buffer pH 7 2 After dehydration in ethanol and embedding in Epon sections were

cut on an LKB Ultrotome and stained with uranyl acetate and lead citrate They were examined at 80 kV in a Siemens Elmiskop or a Philips 300 electron microscope

Fluorescence microscopy The animals were sacrificed and the thyroid with adjacent parts of the trachea oesophagus and surrounding connective tissue was rapidly dissected out. The tissue specimen was frozen in a propane-propylene mixture at the temperature of liquid nitrogen Presence of catecholamines and 5-HT After freeze-drying the specimen was treated with formaldehyde gas (1 h 80°C) embedded in paraffin sectioned at 6 μ and mounted for fluorescence microscopy Catecholamines and 5-HT condense with formaldehyde and under the optical conditions used give rise to a green and a yellow fluorescence respectively (Falck 1962 Falck Hillarp Thieme and Torp 1962 Falck and Owman 1965 Corrodi and Jonsson 1967) Presence of histamine Specimens frozen as above were sectioned at 20 μ in a cryostat (- 30°C) After freeze-drying over P_2O_5 overnight in a desiccator kept in the cryostat the sections were exposed to gaseous o-phthaldialdehyde (OPT) for 90 sec at room temperature followed by hydration for 5 sec They were then mounted for fluorescence microscopy Histamine condenses with OPT giving rise to a blue or yellow fluorophore depending on the concentration of histamine (Ehinger and Thunberg 1967 Håkanson and Owman 1967 Håkanson et al 1970)

Chemical determinations of 5-HT and histamine After sacrificing the animal (rat) the thyroid was dissected out freed from surrounding non-thyroidal tissue under a dissection microscope and weighed In some experiments thyroids were paired The glands were homogenized in an Ultra Turrax homogenizer (Janke and Kunkel KG) in 2 ml 0 4 N perchloric acid. Precipitated proteins were spun down For determination of 5-HT 1 5 ml of the supernatant was extracted with n-butanol as described by Kuntzman et al (1961) and 5-HT was then determined fluorometrically according to Maickel and Miller (1966) For determination of histamine 0 5 ml of the supernatant was extracted with a mixture of n-butanol and chloroform as described by Burkhalter (1962) Histamine was then measured fluorometrically by the method of Shore Burkhalter and Cohn (1959) Amine concentrations were expressed in μg (free base) per g (wet weight) thyroid tissue.

III COMMENTS ON METHODS

Animal care The animals were generally kept in a room with constant temperature (24 ± 1°C) The importance of thermostatic conditions appears from paper 2 In "normal" mice - that is in animals with an intact TSH secretion - thyroid activity was increased upon lowering of environmental temperature and vice versa On the other hand temperature variations did not evoke any obvious alterations in thyroid activity when TSH secretion had been eliminated by the previous administration of thyroid hormone ("T_4-blocked mice" see below) or by hypophysectomy (paper 2) In experiments on such animals the influence of minor temperature variations could hence be disregarded

As appears from paper 3 various exteroceptive stimuli among them injections caused an acute stress response which included an inhibition of thyroid activity secondary to an inhibition of TSH secretion (paper 3 cf Ducommun et al 1966 Salaman 1966) This reaction is important since it is likely to interfere with the thyroid response to any compound given by injection in normal (previously untreated) animals (cf papers 3 4 and 8) By repeated exposures to acute stress however the animal could be adapted to the stressful stimulus so that its influence was minimized (paper 3 cf Ducommun et al 1967) Finally the influence of stress like that of changes in environmental temperature could be disregarded when TSH secretion had been eliminated (T_4-blocked or hypophysectomized mice) (paper 3)

Interpretation and expression of blood and plasma ^{131}I. Under normal conditions about 90% of the plasma ^{131}I was protein-bound (PB ^{131}I) and both blood ^{131}I and PB ^{131}I were eliminated in normal mice with a biological half life of about 5 days (papers 1 and 2) It was assumed that the PB ^{131}I mainly reflected circulating thyroid hormone and the non protein-bound ^{131}I (NPB ^{131}I) was considered to represent inorganic iodide (paper 2 cf Tata 1964) After elimination of surplus iodide the NPB ^{131}I fraction followed a pattern similar to that of PB ^{131}I probably because the former was derived from deiodination of the latter (paper 2) However when the blood levels of corticosteroids deviated extremely from normal (as after hypophysectomy or after administration of such steroids) NPB ^{131}I levels could be markedly changed without any accompanying alteration in the PB ^{131}I (paper 2) Therefore the PB ^{131}I gives a better estimate of the level of circulating thyroid hormone than does the blood ^{131}I (paper 2) although the latter would usually be sufficient

Blood radio-iodine determinations are routinely used in the bioassay of TSH in which the response is measured as the increase in blood radio-iodine two hours after the intravenous injection of the hormone in T_4-blocked mice (paper 1 McKenzie 1958 1960 Sakiz and Guillemin 1964 cf Florsheim et al 1970) In the original work by McKenzie (1958) the percentage ratio of final/initial cpm values makes the parameter however since then various metametric transformations have been introduced (cf paper 1) In the present studies a logarithmic expression was generally employed: response = log (final/initial values x 100) = log % cpm ratio (cf paper 1 Levy et al 1965) The heterogeneity of variance which is inherent in the original parameter was thereby reduced (paper 1 cf Sakiz and Guillemin 1964 Levy et al 1965 Becktel 1967) It should be emphasized in this context that the use of log final cpm covariance adjusted for log initial cpm (Sakiz and Guillemin 1964) is not only unnecessarily laborious (paper 1 Levy et al 1965 Becktel 1967) but may actually be inappropriate (paper 1)

The fact that a blood ^{131}I increase is the parameter of the TSH bioassay does not of course signify that a blood ^{131}I and/or PB^{131}I increase can be evoked only by TSH any stimulus inducing secretion of hormone from prelabelled thyroids would cause such a response Moreover increments in blood radio-iodine levels may be derived even from extrathyroidal sources (Florsheim et al 1970 Solomon et al 1970) However the demonstration of a blood ^{131}I and/or a PB ^{131}I increase in conjunction with signs of endocytosis of thyroglobulin in thyroid follicle cells should suffice to conclude that thyroid hormone secretion has been induced (papers 6 8 11 and 12)

State of thyroid activity in experiments It is important to note that in the majority of the reported experiments TSH secretion had been eliminated by exogenous L-thyroxine (T_4 20 or 2 x 20 μg) or by hypophysectomy These pretreatments reduced the PB ^{131}I to equally low levels indicating that T_4 at the dosage used suppressed TSH secretion completely (paper 2) Mice thus pretreated with T_4 are referred to as "T_4-blocked mice" and the expression "basal conditions" refers to the state of thyroid activity in animals in which TSH secretion had been eliminated (by T_4 or by hypophysectomy) Important advantages are obtained by these procedures Thus when the influence of endogenous TSH is eliminated the effects of various exteroceptive stimuli which normally affect the thyroid via actions on TSH secretion can be disregarded (for example temperature variations and non specific stress see above) At the same time thyroid sensitivity to secretory stimuli is increased about 100 1 000 fold (papers 1 and 2)

In fact thyroid sensitivity and state of activity seem to be in versely related (paper 8)

IV RESULTS AND DISCUSSION

A Influence of TSH and environmental factors on thyroid hormone secretion

In normal mice as well as in T_4-blocked or hypophysectomized ani mals exogenous TSH induced an increase in blood ^{131}I and PB ^{131}I levels (papers 1 2 4 – 8) These responses were preceded by a formation of colloid droplets in thyroid follicle cells (papers 6 and 8) Various factors expected to influence endogenous TSH were also tested As already mentioned, administration of T_4 as well as hypo physectomy reduced blood ^{131}I and PB ^{131}I levels in normal mice Acute exposure of normal mice to cold or heat evoked increased and decreased blood ^{131}I and PB ^{131}I levels respectively (paper 2) and a decrease was also seen following acute stressful procedures (paper 3) The latter response could be minimized by repeated ex posures to the procedure in question (paper 3) No environmental stimulus was effective when TSH secretion had been eliminated (papers 2 and 3) and this supports the assumption that the effects of such stimuli were caused by actions on TSH secretion probably via neural pathways (papers 2 and 3)

These results are consonant with the established concept of the regu lation of thyroid hormone secretion TSH stimulates this secretion and thyroid hormone exerts a negative feed-back effect on the secre tion of TSH (v Euler and Holmgren 1956 a b Reichlin 1963) Extero ceptive stimuli influence TSH secretion by actions on its central nervous control (Brown-Grant 1956 Bottari 1957 Andersson et al 1963 a b 1965 Reichlin 1963 Ducommun et al 1966 1967 Salaman 1966)

B Actions and interactions of amines TSH and adrenergic blocking drugs on thyroid hormone secretion

The fact that various amines are present in the thyroid fostered the idea of an aminergic control of thyroid hormone secretion (papers 4 – 12) The influence of catecholamines and 5-HT on thyroid activity has been the subject of many previous investigations and reviews (see Söderberg 1958 1959 Harrison 1964 Waldstein 1966) but there

is no uniform concept of this influence Both stimulatory and inhibitory effects have been reported (Brown-Grant and Gibson 1956 Ackerman and Arons 1958 Söderberg 1958 1959 Földes et al 1963 Szántó et al 1966) as well as the absence of effects (Brown and Munro 1967 Falconer 1967 Pastan and Wollman 1967) and the prevalent view is that the influence of catecholamines on the thyroid is restricted to actions on the glandular blood flow (Harrison 1964 Ahn et al 1969) There have been great differences in experimental conditions and it is noteworthy that in several investigations thyroid activity must have varied during the experiments partly due to the influence of stress (papers 3 and 8 see also above) and partly due to effects of amines on the secretion and distribution of TSH (cf v Euler and Holmgren 1956 a Söderberg 1958 1959 Harrison 1964) In the present investigations the influence of amines was studied both under "basal conditions and TSH stimulated conditions and it was found that the state of thyroid activity before administration of the test compounds was of extreme importance for the response (paper 8)

In T_4-blocked mice and in hypophysectomized mice (that is during "basal conditions" as defined above cf papers 2 4 - 6 8 and 12) 5 HT (0 1 - 1 0 μmole) and DA (0 5 - 2 0 μmoles) as well as their immediate precursors L-5-hydroxytryptophan (L-5-HTP 6 0 - 10 0 μ moles) and L-dihydroxyphenylalanine (L-DOPA 6 0 - 10 0 μmoles) induced an increase in the blood ^{131}I and PB ^{131}I levels (paper 4) The effects of 5-HT and DA were augmented by pretreatment with a monoamine oxidase inhibitor (nialamide) and the effects of their amino acid precursors L-5-HTP and L-DOPA were abolished by pretreatment with N^1-(DL-seryl)-N^2-(2 3 4-trihydroxybenzyl)hydrazine (Ro 4-4602) an inhibitor of aromatic amino acid decarboxylase (paper 4 Pletscher and Gey 1963 Burkard Gey and Pletscher 1964) This suggests that the amines as such affected the blood ^{131}I and the PB ^{131}I and that their immediate precursors and their deaminated metabolites had little or no influence (paper 4) L-Adrenaline (A 0 01 - 0 05 μmole) L-noradrenaline (NA 0 01 0 05 μmole) L-isoprenaline (IPNA 0 005 - 0 1 μmole) tryptamine and tyramine (5 0 μmoles of each) evoked similar responses (papers 5 6 and 8) Thus several aromatic monoamines were effective whereas the diamine histamine and the aliphatic monoamine acetylcholine were ineffective even in near-lethal doses (paper 5 unpublished observations)

The effect of the amines was very similar to that of a small dose of TSH both in T_4-blocked mice and in hypophysectomized mice

(papers 4 - 6 and 8) and was like the response to TSH preceded by colloid droplet formation and other signs of endocytosis of thyroglobulin in thyroid follicle cells (papers 6 and 8) In normal mice no increase in blood ^{131}I or PB ^{131}I was recorded upon the administration of any of the amines nor upon a small dose of TSH (papers 4 - 6) On these grounds it was concluded that under basal conditions catecholamines and 5 HT can like TSH induce secretion of thyroid hormone by an action within the thyroid (papers 4 - 6 and 8)

Although the vasoactive amines (NA A IPNA and 5-HT) certainly affect thyroid circulation (Söderberg 1958 1959 Mowbray and Peart 1960 Harrison 1964 Falconer 1967 Ahn et al 1969) their thyroid-stimulating actions can for the following reasons hardly be secondary to vascular effects Changes outside the thyroid ought to have produced similar responses in normal mice T_4-blocked mice and hypophysectomized mice but this was not the case (papers 4 and 5) It is also unlikely that a haemodynamic action within the gland was responsible since some of the amines constrict and some dilate thyroid vessels (Söderberg 1958 1959 Mowbray and Peart 1960 Falconer 1967 Ahn et al 1969) Moreover catecholamines and 5-HT stimulate the synthesis of thyroid hormone in isolated thyroid cells (Maayan and Ingbar 1968 1970 Maayan Miller and Ingbar 1971) Thus it seems likely that these amines have like TSH a direct stimulatory action on thyroid follicle cells leading to an increase in both the secretion and the synthesis of thyroid hormone

The observation that the thyroid stimulating properties of the four monoamines were not a consequence of their vasoactive capacities does not exclude the possibility that they modify the thyroid secretory response to TSH by actions on thyroid (or other) blood vessels (cf Söderberg 1958 1959 Mowbray and Peart 1960 Harrison 1964 Falconer 1967 Ahn et al 1969) When given five minutes before TSH NA and A both of which are capable of constricting thyroid blood vessels (Söderberg 1958 1959 Mowbray and Peart 1960 Falconer 1967 Ahn et al 1969) diminished the increase of blood ^{131}I in response to the hormone while no such effect was seen with 5-HT or IPNA (paper 8) which seem to be thyroid vasodilators (Söderberg 1958 1959 Innes and Nickerson 1965) Therefore A and NA apart from their thyroid-stimulating properties may exert an inhibitory effect on thyroid stimulation by TSH by diminishing both the access of TSH to the thyroid and the outflow of thyroid hormone from the gland (paper 8 cf Söderberg 1958 1959 Harrison 1964 Falconer 1967 Ahn et al 1969)

The thyroid-stimulating effects – both on the secretion and the synthesis of hormone – of 5 HT DA A and NA were abolished by the alpha adrenergic blocking agents phentolamine and phenoxybenzamine (papers 5 and 6 Maayan and Ingbar 1968 1970 Maayan Miller and Ingbar 1971 Melander to be published) *whereas the hormone-releasing action* of IPNA was prevented by a beta receptor blocker (L-propranolol) (papers 5 – 7) Alpha blocking agents also inhibited the thyroid secretory responses to TSH and to LATS ("long acting thyroid stimulator a thyroid stimulating factor found in plasma of patients with thyrotoxicosis see McKenzie 1967) while L-propranolol had no such effect (paper 7) Thus it seems probable that the thyroid-stimulating actions of 5 HT DA A and NA as well as that of TSH and LATS involve a follicle cell receptor which in conventional terms is similar to the alpha adrenergic receptor while IPNA produces a similar stimulation although this effect appears to be mediated by beta adrenergic receptors (papers 5 – 7)

In T_4-blocked mice the thyroid secretory responses to various amines (5-HT NA A and IPNA) and to TSH were additive when the substances were administered simultaneously whereas the amines and TSH were mutually antagonistic when given with an interval of two hours (paper 8) This led to the following interpretation Under "basal conditions each of these amines stimulates the secretion of thyroid hormone by a direct action on the thyroid follicle cell Once the gland starts to secrete its sensitivity to secretory stimuli is decreased until "basal conditions are re-established From this point of view all four thyroid stimulating amines might be regarded as potential inhibitors of thyroid stimulation by TSH The results also indicate that TSH and the amines may evoke similar reactions *in the follicle cell even if they differ in their initial inter* actions with this cell (papers 7 and 8) This is supported by the findings that the thyroid secretory responses to both 5-HT A and IPNA (paper 5) as well as to TSH (Bastomsky and McKenzie 1967) are augmented by theophylline pretreatment and that adenyl cyclase activity in isolated thyroid cells is stimulated by A DA 5-HT and TSH (Maayan and Ingbar 1970 Maayan Miller and Ingbar 1971 Maayan personal communication)

In contrast to the findings in T_4-blocked mice and in hypophysectomized mice a decrease in the blood ^{131}I levels was seen upon administration of NA A and 5-HT to normal stress adapted mice (paper 8) This response could well result from an amine TSH interaction in the thyroid as discussed above but it could also

be explained by an amine influence on the secretion and/or the distribution of TSH to and within the gland (paper 8)

In conclusion Catecholamines and 5-HT may affect thyroid activity in several ways and by different actions on the follicle cell on thyroidal and other blood vessels on TSH secretion and on its central nervous control Under "basal conditions" catecholamines and 5-HT can like TSH induce secretion of thyroid hormone by a direct action on the thyroid follicle cell The amines can inhibit the action of TSH by reducing the responsiveness of the gland to TSH TSH and the amines may evoke similar reactions in the follicle cell even if they differ in their initial interactions with this cell When ad ministered their net effects depend on the pre-administration state of thyroid activity

C Physiological significance of amines in thyroid hormone secretion

The presence of arylethylamines within the thyroid suggests that the recorded effects of catecholamines and of 5 HT on thyroid hormone secretion may have physiological significance (papers 4 - 6 and 8) NA is present in intrathyroidal sympathetic terminals (Falck et al 1964 a b Larson Owman and Sundler 1966) and DA is found in thyroid mast cells of ruminants (Falck et al 1964 a) and in bovine C cells (Sundler personal communication) 5-HT is stored in thyroid mast cells of rats and mice (Clayton and Masuoka 1968 papers 9 and 10) and in C cells of sheep and goats (Falck et al 1964 b Falck and Owman 1968) Histamine appears in thyroid mast cells of several species (Selye 1965 Pastan and Almqvist 1966 papers 9 and 10)

The C cells are capable of decarboxylating aromatic monoamino acids such as L-5-HTP and L-DOPA to the corresponding mono-amines such as 5-HT and DA (Larson Owman and Sundler 1966 Falck and Owman 1968 Owman and Sundler 1968) The fact that L-5-HTP and L-DOPA have a thyroid stimulating action but only after their decarboxylation to 5-HT and DA may suggest the existence of a follicle cell C cell interrelation (paper 4) moreover Owman and Sundler (1968) have shown that the concentration of amines within the C cells may be influenced by TSH However subsequent investi gations aiming at a clarification of the follicle cell C cell relation have so far not given conclusive results The present studies were therefore focused on the role of thyroid mast cells (paper 9 11) and of sympathetic nerves (paper 12) in thyroid hormone secretion

a) Amine-containing thyroid mast cells in thyroid hormone secretion

In normal mice intrathyroidal mast cells are extremely few (paper 9) However hypersecretion of TSH - induced by the administration of propylthiouracil (PTU) stimulated the appearance of 5-HT- and histamine-containing mast cells within but not outside the thyroid (paper 9) Their number was decreased upon a reduction of the plasma TSH level following withdrawal of PTU and it was shown that TSH rather than PTU was responsible (paper 9) In rats which normally have higher plasma TSH levels than mice (papers 1 9 and 10) numerous 5-HT- and histamine-storing thyroid mast cells were found already under normal conditions (paper 10 cf Santini 1962 Pastan and Almqvist 1966 Clayton and Szego 1967 Clayton and Masuoka 1968) and hypersecretion of TSH increased their number (paper 10 cf Santini 1962) Thus a correlation between plasma TSH level and the number of thyroid mast cells is indicated (papers 9 and 10)

Within 15 minutes after an injection of TSH to rats the concentrations of 5 HT and histamine in mast cells in the thyroid but not in other tissues were reduced (paper 10 cf Clayton and Szego 1967 Clayton and Masuoka 1968) This was associated with a decrease in their metachromatic reaction which may imply that their content of heparin had also been reduced (paper 10) Similar reactions seemed to occur in mice (paper 9) and in both rats and mice a long term elevation of plasma TSH was accompanied by reduced concentrations of 5-HT and histamine in thyroid mast cells whereas the number of these cells was increased (papers 9 and 10 see above) Neither short-term nor long-term increments in plasma TSH produced any overt ultrastructural signs of degranulation or other changes in the morphology of the mast cells (paper 10) On the basis of these findings it was concluded that in rats and in mice TSH stimulates the appearance of mast cells in the thyroid and induces a release of 5-HT and histamine from these cells (papers 9 and 10) This process takes place without any detectable degranulation (paper 10) and it is restricted to mast cells within the thyroid. Thus activation of thyroid mast cells seems to constitute a part of the thyroid response to TSH and there is a distinct possibility that thyroid mast cells may be involved in the regulation of thyroid hormone secretion (papers 9 and 10 cf Clayton and Szego 1967 Clayton and Masuoka 1968)

In order to obtain evidence for such an involvement an investigation was made on the thyroid secretory response to short term and long term treatment with a liberator of mast cell content compound 48/80 (paper II) In T_4-pretreated rats a single intravenous injection of compound 48/80 (150 300 μg) induced a formation of colloid droplets in thyroid follicle cells and an increase in the blood ^{131}I levels In the thyroids from these animals the mast cells were depleted of 5-HT histamine and of metachromatic material In T_4 pretreated mice no blood ^{131}I increase was recorded upon injection of 48/80 and no or very few intrathyroidal mast cells were detected irrespective of whether 48/80 had been given or not These results suggest that in the rat a secretion of thyroid hormone can be initiated by a substance or substances released from mast cells within the thyroid gland (paper II)

It is known that a variety of substances are released from mast cells upon treatment with 48/80 (Uvnäs 1964 Pastan and Almqvist 1966 Fillion Slorach and Uvnäs 1970) and anyone of these might be responsible for the thyroid activation Among the substances known to be released 5-HT was found to induce an increase in the level of blood ^{131}I of the rat (paper II) as well as in the mouse (papers 4 - 6 and 8 see also above) whereas histamine gave no such response in either species (papers 5 and II) As mentioned above 5-HT can stimulate the follicle cell by a direct action therefore the substance which when released from the mast cells activated the follicle cells could be 5-HT and is presumably not histamine (paper II)

The importance of mast cells for thyroid hormone secretion need not be restricted to actions of mast cell content(s) directly on the follicle cell The TSH induced release of 5-HT and of histamine from thyroid mast cells (papers 9 and 10 see above) can be the cause of the increase in thyroid blood flow that is part of the thyroid response to TSH (Hoffman and Levey 1965 Clayton and Szego 1967 Clayton and Masuoka 1968) although which substance is the mediator of this vascular response is still an open question (10) Furthermore thyroid mast cells contain an alkaline protease which is capable of hydrolyzing thyroglobulin (Pastan and Almqvist 1966) If released this enzyme could cause an extracellular hydrolysis of thyroglobulin that has escaped intracellular degradation (paper II)

If thyroid mast cells do play a role in the process of thyroid hormone secretion chronic depletion of mast cell contents could be expected

to affect the thyroid secretory response to TSH The investigation (paper 11) showed that the increase of blood ^{131}I in response to TSH was smaller in rats in which the mast cells had been depleted by repeated 48/80 injections than in controls In mice which normally have very few intrathyroidal mast cells no such difference was noted It can be assumed therefore that the diminished secretory response in the rat thyroid was due to the absence of mast cells within the gland and that in this species thyroid mast cells are in volved in the regulation of thyroid hormone secretion (paper 11) On the other hand thyroid mast cells cannot be indispensable in this process since secretory responses to TSH were obtained in their absence Their function in thyroid hormone secretion therefore seems to be facilitatory in nature The appearance of thyroid mast cells in mice upon TSH hypersecretion (paper 9) may suggest that this facilitatory function is a general feature at least when the secretory activity of the thyroid is high (paper 11)

<u>In conclusion</u> In mice and rats TSH induces the appearance of 5–HT– and histamine–containing mast cells within the thyroid and promotes the release of these amines and probably other sub stances as well from these cells Substances thus released stimu late and facilitate thyroid hormone secretion by direct actions on the follicle cells and on the thyroid blood vessels

b) Thyroid hormone secretion induced by sympathetic stimulation

The possibility of a control by the sympathetic nervous system of thyroid activity has been a matter of great and long standing interest (for review see Harrison 1964 Waldstein 1966) As early as 1865 overactivity of the cervical sympathetic nerves was suggested as a cause of hyperthyroidism (Reith 1865 cf Harrison 1964) Lacking convincing experimental support however the idea of a direct sympathetic influence on thyroid activity was abandoned by most investigators and the prevalent view is that the sympathetic innervation of the thyroid gland is physiologically important only for the regulation of thyroid blood flow (cf Harrison 1964)

As previously shown and discussed (sections III IV B and paper 8) the effect of a stimulus on the secretion of thyroid hormone is dependent upon the preceding secretory activity and also modifies the responsiveness of the thyroid gland to another stimulus Most experiments concerning sympathetic influences on thyroid activity

have been performed on animals with an intact TSH secretion (cf Harrison 1964) that is the sensitivity of the thyroid has been low and a variety of environmental stimuli may have affected the response by actions on TSH secretion (cf sections III IV A and B papers 2 3 and 8) Furthermore the choice of parameters has usually been restricted to (radio-)iodine levels in the thyroid and in the blood few studies seem to have concerned colloid droplet formation in the follicle cells and no study has related the response obtained to the distribution of sympathetic terminals within the gland In the present investigation (paper 12) the influence of sympathetic stimulation on thyroid hormone secretion was studied under "basal conditions both colloid droplet formation and blood ^{131}I levels were measured and the findings were correlated with the distribution of catecholamine-containing nerve terminals within the thyroid gland (paper 12)

In T_4-blocked mice stimulation of the right cervical sympathetic trunk induced a formation of colloid droplets in thyroid follicle cells and an increase in blood ^{131}I indicating secretion of thyroid hormone (paper 12) The droplet formation was restricted to follicle cells in the thyroid regions supplied by this nerve – the right lobe the isthmus and the medial part of the left lobe – as judged from the distribution of catecholamine-containing nerve terminals of animals with a left side sympathectomy (paper 12) This distribution shows that the stimulatory effect resulted from an intrathyroidal action since any extrathyroidal effect ought to have produced signs of endocytosis within the entire gland (paper 12)

Such signs on the other hand were observed upon administration of NA which as in previous studies (section B papers 5 6 and 8) also evoked a blood ^{131}I increase The effect of sympathetic stimulation and of exogenous NA were inhibited by the alpha adrenergic blocking agent phentolamine Together these findings should allow the suggestion that the thyroid activation following sympathetic stimulation was induced by NA released from intrathyroidal sympathetic nerve terminals (paper 12)

The intimate relation of sympathetic terminals to the arterial supply of various organs together with the evidence that exogenous NA can cause vasoconstriction in the thyroid (Söderberg 1958 1959 Mowbray and Peart 1960 Ahn et al 1969) could lead to the opinion that the changes observed in this study were secondary to a reduction in thyroid blood flow (cf Harrison 1964) However this explanation

appears unlikely for the following reasons 1) Aromatic monoamines induce secretion of thyroid hormone irrespective of whether they have vasoconstrictory or vasodilatatory properties (papers 4 – 6 and 8 see above) 2) there is no direct relation between secretion rate and blood flow in the thyroid (Söderberg 1958 1959) 3) a direct stimulatory effect of NA on thyroid follicle cells has been recorded (Maayan and Ingbar 1968) 4) neither uni- nor bilateral occlusion of the carotid blood flow evokes secretion of thyroid hormone (paper 12) Therefore it is suggested that under "basal conditions" sympathetic stimulation induces secretion of thyroid hormone through a direct action on the thyroid follicle cells by NA released from intrathyroidal sympathetic nerve terminals (paper 12)

The various interactions between exogenous TSH and NA (and other thyroid-stimulating amines) described above (section B paper 8) imply that complex interactions may exist between endogenous TSH and the sympathetic nervous system with respect to the regulation of thyroid activity It seems appropriate to re introduce the hypothesis that an excessive sympathetic activity may promote hyperthyroidism The progress made in the treatment of this disease with adrenolytic drugs such as reserpine guanethidine and adrenergic blocking agents (cf Canary et al 1957 Waldstein 1966 Dillon et al 1970) may support this hypothesis

In conclusion In mice sympathetic stimulation can induce secretion of thyroid hormone through a direct action on the follicle cells by NA released from intrathyroidal sympathetic nerve terminals Thyroid hormone secretion may be regulated by interactions between sympathetic and thyrotropic stimulation and it is possible that sympathetic overactivity is involved in the development of hyperthyroidism

V SUMMARY

The actions and interactions of TSH and arylethylamines on thyroid hormone secretion were investigated in mice and rats by a combination of chemical histochemical light and electron microscopic and bioassay procedures The following suggestions and conclusions were made

Catecholamines and 5-hydroxytryptamine may affect thyroid activity in several ways and by different actions on the follicle cells on thyroidal and other vessels on TSH secretion and on its central nervous control Under "basal conditions" (that is when TSH secretion has been eliminated) catecholamines and 5 hydroxytryptamine can like TSH induce secretion of thyroid hormone by a direct action on the thyroid follicle cell The amines can also inhibit the action of TSH by reducing thyroid sensitivity TSH and the amines probably evoke similar reactions in the follicle cell even if they differ in their initial interactions with this cell When administered their net effects depend on the pre-administration state of thyroid secretory activity The observations indicate that intrathyroidal arylethylamines could under physiological conditions participate in this secretory process

In both rats and mice TSH stimulates the appearance of 5-hydroxy tryptamine- and histamine-containing mast cells within the thyroid and promotes the release of these amines and probably other substances as well from these cells Substances thus released facilitate thyroid hormone secretion by actions on the follicle cells and on thyroid vessels

In mice sympathetic stimulation can induce secretion of thyroid hormone through a direct action on the follicle cells by noradrenaline released from intrathyroidal nerve terminals Thyroid hormone secretion may be regulated by complex interactions between sympathetic and thyrotropic stimulation and it is possible that sympathetic overactivity is involved in the development of hyper thyroidism

VI ACKNOWLEDGMENTS

I wish to express my sincere gratitude to
Professor Stephen Thesleff head of this department who kindly placed laboratory facilities to my disposal and who has read and critisized the manuscript with great care to
Drs Claus Rerup Rolf Håkanson and Erik Nilsson this department Christer Owman and Frank Sundler Department of Histology University of Lund and Lars Ericson Department of Anatomy University of Göteborg who have all been teachers and collaborators at the same time to
Dr John Harris Department of Neurology University of Newcastle School of Medicine for his careful reading and correction of the manuscript and papers to
Mrs Lena Kvist Miss Siw Erman Miss Anita Åkesson Miss Ann-Kristin Helander and Mrs Karin Irenesson for their skilful technical assistance and to
The Medical Faculty University of Lund Nordisk Insulinfond AB Ferrosans Jubileumsstiftelse and Svenska Livförsäkringsbolags Nämnd för Medicinsk Forskning for financial support of the investigations

VII REFERENCES

ACKERMAN N B and W L. ARONS The effect of epinephrine and norepinephrine on the acute thyroid release of thyroid hormones Endocrinology 1958 62 723 737

AHN C S J C ATHANS and I N ROSENBERG Effects of epinephrine and of alteration in glandular blood flow upon thyroid function studies using thyroid vein cannulation in dogs Endocrinology 1969 84 501 - 507

ANDERSSON B A H BROOK and L EKMAN Further studies of the thyroidal response to local cooling of the heat loss center Acta physiol scand 1965 63 186 - 192

ANDERSSON B L EKMAN C C GALE and J W SUNDSTEN Control of thyrotrophic hormone (TSH) secretion by the "heat loss center Acta physiol scand 1963 (a) 59 12 33

ANDERSSON B C C GALE and A OHGA Suppression by thyroxine of the thyroidal response to local cooling of the 'heat loss center Acta physiol scand 1963 (b) 9 67 - 73
AREY L B Developmental Anatomy Saunders Philadelphia 19"0 231 - 251
BASTOMSKY C H and J M McKENZIE Cyclic AMP a mediator of thyroid stimulation by thyrotropin Amer J Physiol 1967 213 753 - 758
BECKTEL J M Comparison of ratio and covariance analysis of TSH assays Proc Soc exp Biol Med. 1967 124 999 - 1000
BOTTARI P M The concentration of thyrotropic hormone in the blood of the rabbit under different experimental conditions Ciba Found Coll Endocr 1957 11 52 - 72
BOWERS C Y A V SCHALLY G A REYNOLDS and W D HAWLEY Interactions of L-thyroxine or L-triiodothyronine and thyrotropin releasing factor on the release and synthesis of thyrotropin from the anterior pituitary gland of mice Endocrinology 1967 81 741 - 747
BOWERS C Y K L LEE and A V SCHALLY A study of the interaction of the thyrotropin releasing factor and L-triiodothyronine: Effects of puromycin and cycloheximide Endocrinology 1968 82 75 - 82
BOYD J D Development of the human thyroid gland. In The thyroid gland ed R Pitt-Rivers and W R. Trotter Butterworths London 1964 9 - 31
BROWN J and D S MUNRO A new in vitro assay for thyroid stimulating hormone J Endocr 1967 38 439 - 449
BROWN-GRANT K Changes in thyroid activity of rats exposed to cold J Physiol 1956 131 52 57
BROWN-GRANT K and J G GIBSON The effect of exogenous and endogenous adrenaline on the uptake of radio-iodine by the thyroid gland of the rabbit J Physiol 1956 131 85 - 101
BURKARD W P K F GEY and A PLETSCHER Inhibition of decarboxylase of aromatic amino acids by 2 3 4 trihydroxy benzylhydrazine and its seryl derivative Arch Biochem Biophys 1964 107 187 196
BURKHALTER A The formation of histamine by fetal rat liver Biochem Pharmacol 1962 11 315 322
CANARY J J M SCHAAF B J DUFFY Jr and L H KYLE Effects of oral and intramuscular administration of reserpine in thyrotoxicosis New Engl J Med 1957 257 435 442

CLAYTON J A and D T MASUOKA TSH-induced mobilization of serotonin from perivascular mast cells in the rat thyroid Endocrinology 1968 83 263 271
CLAYTON J A and C M SZEGO Depletion of rat thyroid serotonin accompanied by increased blood flow as an acute response to thyroid-stimulating hormone Endocrinology 1967 80 689 - 698
COPP D H Calcitonin and parathyroid hormone Annu Rev Pharmacol 1969 9 327 - 344
COPP D H Endocrine regulation of calcium metabolism Annu Rev Physiol 1970 32 61 86
CORRODI H and G JONSSON The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines J Histochem Cytochem 1967 15 65 - 78
DILLON P T J BABE C R MELONI and J J CANARY Reserpine in thyrotoxic crisis New Engl J Med 1970 283 1020 - 1023
DUCOMMUN P E SAKIZ and R GUILLEMIN Lability of plasma TSH levels in response to nonspecific exteroceptive stimuli Proc Soc exp Biol Med 1966 121 921 - 923
DUCOMMUN P W WALE E SAKIZ and R GUILLEMIN Reversal of the inhibition of TSH secretion due to acute stress Endocrinology 1967 80 953 - 956
EHINGER B and R THUNBERG Induction of fluorescence in histamine-containing cells Exp Cell Res 1967 47 116 - 122
EKHOLM R and S SMEDS On dense bodies and droplets in the follicular cells of the guinea pig thyroid J Ultrastruct Res 1966 16 71 - 82
EKHOLM R and T ZELANDER Nerve tissue in the mouse thyroid gland Proc Europ Reg Conf on Electron Microscopy Delft 1960 Vol II 827 - 830
v EULER C and B HOLMGREN The thyroxine "receptor" of the thyroid pituitary system. J Physiol 1956 (a) 131 125 - 136
v EULER C and B HOLMGREN The role of hypothalamo-hypophysial connexions in thyroid secretion J Physiol 1956 (b) 131 137 146
FALCK B. Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta physiol scand 1962 56 Suppl 197
FALCK B N Å HILLARP G THIEME and A TORP Fluorescence of catecholamines and related compounds condensed with formaldehyde J Histochem Cytochem 1962 10 348 354

FALCK B B LARSON C v MECKLENBURG F ROSENGREN and K SVENAEUS On the presence of a second specific cell system in mammalian thyroid gland. Acta physiol scand 1964 (a) 62 491 - 492

FALCK B T NYSTEDT E ROSENGREN and J STENFLO Dopamine and mast cells in ruminants Acta pharmacol toxicol 1964 (b) 21 51 58

FALCK B and CH OWMAN A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines Acta Univ Lundens (II) 1965 7 1 - 23

FALCK B and CH OWMAN 5-Hydroxytryptamine and related amines in endocrine cell systems Advances Pharmacol 1968 6 (A) 211 - 231

FALCONER I R The effect of adrenaline and noradrenaline on hormone secretion and blood flow from the thyroid vein in sheep with exteriorized thyroids J Physiol 1967 188 425 - 434

FALCONI G and G L ROSSI Transauricular hypophysectomy in rats and mice Endocrinology 1964 74 301 - 303

FILLION G M B S A SLORACH and B UVNAS The release of histamine heparin and granule protein from rat mast cells treated with compound 48/80 in vitro Acta physiol scand 1970 78 547 - 560

FLORSHEIM W H A D WILLIAMS and E SCHÖNBAUM On the mechanism of the McKenzie bioassay Endocrinology 1970 87 881 - 888

FÖLDES J L KRASZNAI and K MEGYESI Effect of nor-adrenaline on the response of the thyroid to thyrotrophic hormone Acta endocr 1963 43 280 - 293

GUILLEMIN R. The adenohypophysis and its hypothalamic control Annu Rev Physiol 1967 29 313 - 348

HARRISON T S Adrenal medullary and thyroid relationships Physiol Rev 1964 44 161 - 185

HOFFMAN A L and H L LEVEY Thyroid histamine levels in control and propylthiouracil treated rats Federation Proc 1965 24 188

HÅKANSON R L JUHLIN CH OWMAN and B SPORRONG Histochemistry of histamine Microspectrofluorometric characterization of the fluorophores induced by o-phthaldialdehyde J Histochem Cytochem 1970 18 93 - 99

HÅKANSON R and CH OWMAN Concomitant histochemical demonstration of histamine and catecholamines in enterochromaffin-like cells of gastric mucosa Life Sci 1967 6 759 766

INNES I R and M NICKERSON Drugs acting on postganglionic adrenergic nerve endings and structures innervated by them (sympathomimetic drugs) In The Pharmacological Basis of Therapeutics ed L S Goodman and A Gilman Macmillan New York 1965 477 - 520 (497)
KOSANOVIĆ M R EKHOLM U STRANDBERG and S SMEDS The effect of TSH on the acid phosphatase and thyroglobulin hydrolyzing activities in the guinea pig thyroid Exp Cell Res 1968 5 147 - 160
KUNTZMAN R P A SHORE D F BOGDANSKI and B B BRODIE Microanalytical procedures for fluorometric assay of brain DOPA 5HTP decarboxylase norepinephrine and serotonin and a detailed mapping of decarboxylase activity in brain J Neurochem 1961 6 226 - 232
LARSON B. CH OWMAN and F SUNDLER Monoaminergic mechanisms in parafollicular cells of the mouse thyroid gland Endocrinology 1966 78 1109 - 1114
LEVY R.P W L McGUIRE R.K SHAW and G E BARTSCH Effect of species differences on the bio-assay of thyrotropin 1965 76 890 - 894
MAAYAN M L. and S H INGBAR Epinephrine Effect on uptake of iodine by dispersed cells of calf thyroid gland Science 1968 162 124 - 125
MAAYAN M L and S H INGBAR Effects of epinephrine on iodine and intermediary metabolism in isolated thyroid cells Endocrinology 1970 87 588 - 595
MAAYAN M L. S L. MILLER and S H INGBAR. Effects of serotonin on iodide and intermediary metabolism in isolated thyroid cells Endocrinology 1971 88 620 - 626
MAICKEL R P and F P MILLER Fluorescent products formed by reaction of indole derivatives and o-phthaldialdehyde Analyt Chem 1966 38 1937 - 1938
McKENZIE J M The bioassay of thyrotropin in serum Endocrinology 1958 63 372 382
McKENZIE J M Bio-assay of thyrotropin in man Physiol Rev 1960 40 398 - 414
McKENZIE J M The long acting thyroid stimulator: Its role in Graves disease Rec Progr Hormone Res 1967 23 1 - 46
MOWBRAY J D and W S PEART Effects of noradrenaline and adrenaline on the thyroid. J Physiol 1960 151 261 - 271
OWMAN CH and F SUNDLER Indole metabolism in thyroid C cells of the mouse Effects of thyrocalcitonin and thiouracil In Calcitonin Proceedings of the symposium on thyrocalcitonin and the C cells ed S Taylor Heinemann London 1968 110 121

PASTAN I and S ALMQVIST Localization of rat thyroid alkaline protease to mast cells Endocrinology 1966 78 361 - 366
PASTAN I and S H WOLLMAN Colloid droplet formation in dog thyroid in vitro Induction by dibutyryl Cyclic-AMP J Cell Biol 1967 35 262 - 266
PITT-RIVERS R and W R TROTTER The thyroid gland. Butterworths London 1964
PLETSCHER A and K F GEY The effect of a new decarboxylase inhibitor on endogenous and exogenous monoamines Biochem Pharmacol 1963 12 223 - 228
PURVES H D Control of thyroid function In The thyroid gland ed R Pitt Rivers and W R Trotter Butterworths London 1964 1 - 38
REICHLIN S Regulation of pituitary thyrotropin release In Thyrotropin ed S C Werner Thomas Springfield Illinois 1963 56 - 79
REITH A Exophtalmosis-enlargement of thyroid gland-death-autopsy affection of cervical sympathetic Med Times Gazette 1865 2 521 523
ROMEIS B Mikroskopische Technik Oldenbourg München 1948 327
SAKIZ E and R GUILLEMIN On a method for calculation and analysis of results in the McKenzie assay for thyrotropin (TSH) Proc Soc exp Biol Med 1964 115 856 - 860
SALAMAN D F The action of stress on plasma TSH concentration in the rat J Physiol 1966 186 134 135 P
SANTINI F Le Mastzellen nella tiroide di ratto e le loro modificazioni in rapporto ai vari stati funzionali della ghiandola Arch Ital Anat Embriol 1962 67 443 458
SCHALLY A V A J KASTIN W LOCKE and C Y BOWERS Hypothalamic releasing and inhibiting factors In Hormones in Blood. ed C H Gray and A L. Bacharach Academic Press London New York. 1967 491 - 525
SCHALLY A V T W REDDING C Y BOWERS and J F BARRETT Isolation and properties of porcine thyrotropin releasing hormone J biol Chem 1969 244 4077 4088
SELJELID R A study of endocytosis in thyroid follicle cells Thesis Tryckeri Balder AB Stockholm 1966
SELYE H The Mast Cells Butterworths Washington D C 1965
SHORE P A A BURKHALTER and V H COHN A method for the fluorometric assay of histamine in tissues J Pharmacol exp. Therap 1959 127 182 186

SOLOMON D H G N BEALL and I J CHOPRA Mobilization of tissue thyroxine in mice by thyroxine-binding proteins studies with a double isotope McKenzie assay Abstract no 62 The Sixth International Thyroid Conference Vienna 1970 Verlag der Wiener medizinischen Akademie 1970 74

STEIN O and J GROSS Metabolism of ^{125}I in the thyroid gland studied with electron microscopic autoradiography Endocrinology 1964 75 787 - 798

SZANTO L A L REVICZKY and T GRYNAEUS Effect of serotonin (5 hydroxytryptamine) on thyroid activity in albino rats Acta physiol Acad Sci hung 1966 29 183 - 194

SÖDERBERG U Short term reactions in the thyroid gland Acta physiol scand 1958 42 Suppl 147

SÖDERBERG U Temporal characteristics of thyroid activity Physiol Rev 1959 39 777 - 810

TATA J R. Distribution and metabolism of thyroid hormones In The thyroid gland ed R. Pitt-Rivers and W R Trotter Butterworths London 1964 163 - 186.

UVNAS B Release processes in mast cells and their activation by injury Ann N Y Acad Sci 1964 116 880 - 890

WALDSTEIN S S Thyroid - catecholamine interrelations Annu Rev Med 1966. 17 123 - 132

WERNER S C Thyrotropin Thomas Springfield Illinois 1963

WETZEL B K S S SPICER and S H WOLLMAN Changes in fine structure and acid phosphatase localization in rat thyroid cells following thyrotropin administration J. Cell Biol 1965 25 593 - 618

WOLLMAN S H S S SPICER and M. S BURSTONE Localization of esterase and acid phosphatase in granules and colloid droplets in rat thyroid epithelium J Cell Biol 1964 21 191 202

SUPPLEMENTUM 371

From the Department of Histology Karolinska Institutet
S-104 01 Stockholm, Sweden

ACTA PHYSIOLOGICA SCANDINAVICA

Stockholm 1971

Acta physiol. scand. 1971 Suppl. 371 1—18
From the Department of Histology Karolinska Institutet, S-104 01 Stockholm 60, Sweden

³H Noradrenaline Release and Mechanical Response in the Field Stimulated Mouse Vas Deferens

By

LARS-OVE FARNEBO and TORBJÖRN MALMFORS

Abstract

FARNEBO, L.-O and T MALMFORS *³H-Noradrenaline release and mechanical response in the field stimulated mouse vas deferens* Acta physiol. scand. 1971 Suppl. 371 1—18

Isolated mouse vas deferens was preincubated with ³H-noradrenaline (³H-NA) and then mounted in a small glass chamber where it was superfused with buffer and stimulated by an lectrical field. Tritium overflow into the buffer and mechanical activity was followed.

Almost all the radioactivity taken up and etained I the vas deferens, and about 75 % of the tritium overflow during stimulation was due t ³H-NA. Stimulation for 30 sec at 4—16 Hz caused biphasic contraction of the vas deferens with an initial rapid twitch and second tetanus-like phase of contraction. The stimulation-induced tritium overflow during stimulation t 16 Hz for 30 sec was 1.8 % (*i.e* 1.8 % of the total tritium content of the vas deferens was overflowing in response to stimulation) The fraction of the tritium content of the vas deferens overflowing *per* impulse at different frequencies of stimulation was the same t 1—16 Hz and was around 3.5×10^{-5} Upon stimulation for various time periods (10—120 sec) the stimulation-induced overflow was found to be proportional to time. The mechanical response, on the other hand, decreased upon prolonged stimulation

No contractions were elicited in vas deferens from mice pretreated with 6-OH-dopamine. Atropine did not affect the response to stimulation. N contraction and no stimulation-induced overflow was obtained when tetrodotoxin was added to the superfusing buffer Inhibition of neuronal uptake of NA by desipramine potentiated the contraction caused by stimulation, but the stimulation-induced overflow was almost unchanged. The α-receptor blocking drugs phentolamine and phenoxybenzamine decreased the second phase of contraction and increased the stimulation-induced overflow Papa erine inhibited the contraction and increased the stimulation-induced overflow Stimulation of α-receptors by methoxamine potentiated the contraction and decreased the stimulation-induced overflow

From the drug-experiments it is concluded that a decreased effector response is accompanied by an increased ³H NA release, while an increased effector response is accompanied by a decreased ³H NA release. The results indicate the existence of a transsynaptic regulatory mechanism for NA release.

Introduction

Stimulation of adrenergic nerves causes release of noradrenaline (NA) from the nerve terminals (see Euler 1956) The NA released can influence receptors on the effector cells and cause response of the effector organ. After activation of the receptors, NA is inactivated either by enzymatic degradation, reuptake into the nerves or overflow into the circulation (see Folkow *et al.* 1967)

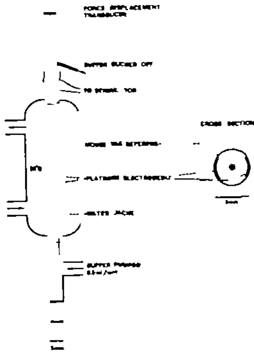


Fig. 1 Experimental set-up. Th mouse vas deferens was mounted in water jacketed (32 C) glass chamber (volume about 0.8 ml) and superfused by oxygenated buffer (0.5 ml/min) The buffer was collected from the top of the chamber by means of suction directly into vials for liquid scintillation counting The upper end of the vas deferens was connected to force-displacement transducer for isometric recording of tension on a polygraph. Two platinum electrodes (3 mm wide) placed parallely on both sides of the vas def rens were connected to a stimulator

The relation between NA overflow and effector response has been studied in various adrenergic neuro-effector systems, mainly smooth muscle organs. Inhibition of NA reuptake into the nerves by drugs such as cocaine or desipramine, potentiates smooth muscle contraction caused by stimulation of sympathetic nerves (Trendelenburg, 1959) Generally an increased NA overflow has been difficult to demonstrate after inhibition of NA uptake (Blakeley *et al.* 1963 Geffen, 1965 Boullin *et al* 1967) although in some cases the increased effector response has been accompanied by an increase of NA overflow (Huković and Muscholl, 1962 Thoenen *et al* 1964a, b)

The contraction of smooth muscle organs caused by stimulation of adrenergic nerves can be inhibited by α-receptor blocking drugs such as phentolamine, phenoxybenzamine or hydergine. These drugs greatly increase NA overflow upon stimulation (Brown and Gillespie 1957 Rosell *et al.*, 1963 Thoenen *et al.* 1964c Boullin *et al* 1967)

Previous studies on the field stimulated rat iris indicated that the above mentioned drugs cause changes in NA *overflow* which are due to changes in NA *release* from the nerve terminals (Farnebo and Hamberger 1970a 1971) speaking in favour of the view f variable transmitter release (see Häggendal, 1970)

These studies were in the present investigation extended to experiments on the direct relation between NA overflow and mechanical response aiming to disclose the possible existence of nerve terminal-effector organ interactions.

The isolated transmurally stimulated mouse vas deferens was used, as it has a rich adrenergic nerve supply does not show spontaneous mechanical activity and is fairly thin permitting good possibilities for diffusion of NA and drugs.

Material and Methods

Albino mice (NMRI 25—30 g) were killed by dislocation of the neck. The vasa deferentia were dissected out by cutting the testicular and prostatic ends and removing the mesenteries close to the organs. The isolated vas deferens was then incubated for 30 min at 37 C in oxygenated (6.5 % CO_2 in O_2) Krebs-Ringer bicarbonate buffer solution (composition in mmoles per litre: NaCl, 133; KCl, 4.7; $CaCl_2$, 2.5; NaH_2PO_4, 1.3; MgSO 1.2 $NaHCO_3$, 16 glucose 8) containing ^{3}H NA 10^{-7} M

After rinsing in fresh buffer for 15 min the vas deferens was mounted in small stimulation chamber (volume 0.8 ml) and connected to a force-displacement transducer (Grass FT03C) see Fig 1 Mechanical activity was recorded isometrically on Grass Model 7B Polygraph. Tension was adjusted to about 500 dy and the vas deferens was then allowed to rest for 60 min during superfusion with oxygenated buffer (0.5 ml/min) It was then stimulated transmurally according to Birmingham and Wilson (1963) for 10—120 sec (usually 30 sec) at 30 min intervals. An electrical field was generated between the platinum electrodes in the chamber by a Grass S-48 stimulator (supramaximal voltage 2 msec, 1—16 Hz) Force development during stimulation was quantitated by means of an electronic integrating device (Grass 7P10B)

For studies on drug effects the vas deferens was stimulated five times for 30 sec at 30 min intervals. During the first two stimulations the organ was superfused by drug-free buffer and during the following three stimulations the drug to be tested was added to the superfusing buffer

The superfusing buffer was collected in 5 min fractions (2.5 ml) directly into counting vials. After the addition of 5 ml Insta-Gel® scintillation solution, total radioactivity was determined by counting in a Packard Model 3320 Liquid Scintillation Spectrometer At the end of the superfusion the vas deferens was solubilized in 0.5 ml Soluene® and radioactivity determined after the addition of 10 ml toluene scintillation solution. Quenching was determined by recounting representative samples after addition of a standard amount of ^{3}H toluene.

The stimulation-induced tritium overflow (in dpm) was calculated from the total tritium overflow by subtracting the estimated spontaneous overflow during the stimulation period. To make comparisons between several consecutive stimulations of the same organ possible stimulation-induced overflow was also calculated as per cent of the total tritium content in the organ at the onset of each stimulation and corrections were thus made for the continuous loss of ^{3}H NA from the organ during superfusion (Farnebo and Hamberger 1970a, 1971; Häggendal et al., 1970)

In some experiments the superfusate was collected in cool 0.4N perchloric acid and the vas deferens homogenized in cool 0.4N perchloric acid. The extracts were analysed for ^{3}H-NA and its ^{3}H-metabolites by ion-exchange chromatography on Dowex 50W X4 columns according to Carlsson and Waldeck, 1963 (for details see Farnebo, 1971) No corrections for recovery were made.

Substances used dl-noradrenaline-7 ^{3}H HCl (5—10 Ci/mmole, Radiochemical Centre, Amersham) l-noradrenaline bitartrate (Sigma) 6-OH-dopamine HCl (Hassle) desipramine (Pertofran® Geigy); phentolamine (Regitin®, Ciba) phenoxybenzamine (Dibenyline® Smith, Kline & French) propranolol (Inderal® ICI), papaverine (ACO) methoxamine HCl (Wellcome); tetrodotoxin (Sankyo), reserpine phosphate (Ciba) atropine sulphate (Sigma) Insta-Gel® and Soluene® (Packard)

Results

Uptake and retention of ^{3}H NA Isolated mouse vas deferens was incubated with ^{3}H NA 10^{-7} M for 30 min, rinsed for 15 min, mounted in the stimulation chamber and superfused for 60 min before stimulation. The tritium content of the vas deferens at the onset of the first stimulation was 129 000 ± 4400 dpm (mean ±

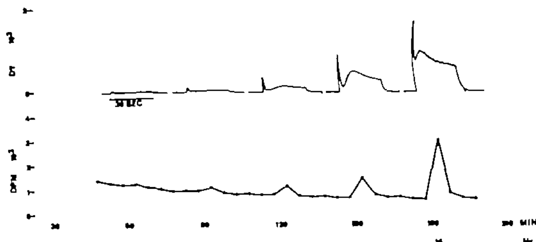


Fig 2. Mechanical response (upper) and tritium overflow (lower) upon stimulation of a mouse vas deferens preincubated with ^{3}H NA. The organ was stimulated at different frequencies for 30 sec every 30 min. The tritium overflow per 5 min fraction is shown from the whole experiment, while mechanical response is illustrated only from the stimulation periods.

S.E.M of 80 experiments) This amount corresponds to about 1 ng of radioactively labelled NA, as almost all radioactivity certainly is due to unchanged ^{3}H NA (see below) The endogenous NA content of the mouse vas deferens is about 6 μg/g (Sjöstrand 1965) and the weight of the isolated organ is about 10 mg (10.2 $\pm$ 0.3 mg mean $\pm$ S E.M. of 8 determinations) which gives an approximative NA content of 60 ng Thus, labelled NA made up about 2 % of the total NA stores in the vas deferens.

Effect of different stimulation frequencies. See Fig 2 and 3 Stimulation at 1 Hz for 30 sec caused very small separated contractions. At 2 Hz a slight summation of the contractions occurred. At 4 Hz a rapid twitch response was initially obtained, followed by a plateau of lower tension. The twitch was increased at 8 Hz and so was also the second phase of contraction. The twitch made up about 20 % of the integrated contraction during stimulation for 30 sec. At 16 Hz the twitch was followed by a vigorous tetanus-like contraction About 10 % of the integrated con

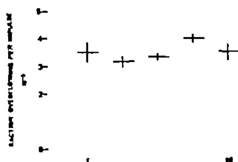


Fig 3 Fraction of the tritium content overflowing per impulse at different frequencies of stimulation from vas deferens preincubated with ^{3}H-NA. Stimulation for 120 sec. Mean $\pm$ S E M of 6 experiments.

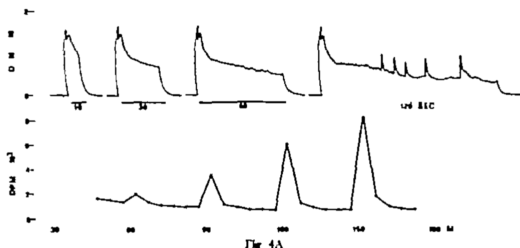


Fig. 4A

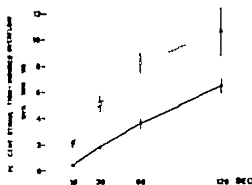


Fig. 4B

Fig. 4. Stimulation of mouse vas deferens preincubated with ³H-NA. The organ was superfused with drug free buffer and four consecutive stimulations were performed at 16 Hz for 10, 30, 60 and 120 sec at 30 min intervals. A. Mechanical response (upper) and tritium overflow (lower) from one experiment. B. Integrated mechanical response (○——○) and per cent stimulation-induced overflow (●——●) (i.e. the stimulation-induced tritium overflow in per cent of the total tritium content of the vas deferens at the onset of each stimulation). Mean ±S.E.M. of 6 experiments.

TABLE I. ³H-NA and its ³H-metabolites in the superfusates and in the vas deferens.

	³H NA %	³H normetanephrine %	³H-deaminated metabolites %
Superfusat from prestimulation period	33±1	44±3	23±4
Superfusate from stimulation period	73±6	20±4	7±5
Vas deferens	98±1	1±1	1±0

Isolated vasa deferentia were incubated with ³H-NA 10⁻⁷M for 30 min, rinsed in drug-free buffer for 15 min and then superfused by drug-free buffer for 45 min. The superfusate from the following 15 min was collected in cool 0.4N perchloric acid as prestimulation sample. The vas deferens was then stimulated at 16 Hz for 120 sec and the superfusate from this period collected as stimulation sample. At the end of the procedure the vas deferens was homogenized in cool 0.4N perchloric acid. The samples were assayed for ³H-NA, ³H-normetanephrine and ³H-deaminated metabolites by ion-exchange chromatography as described in Material and Methods. The figures are expressed as per cent of the total ³H-radioactivity recovered. Mean ±S.E.M. of 4 determinations.

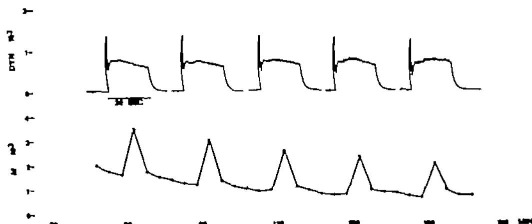
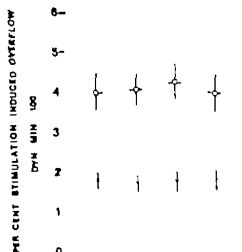


Fig 5A



NO DRUG

Fig 5B

Fig. 5 Stimulation of mouse vas deferens preincubated with ^{3}H NA. The organ was superfused with drug-free buffer and five consecutive stimulations were performed at 16 Hz for 30 sec at 30 min intervals. A. Mechanical response (upper) and tritium overflow (lower) from the same experiment. B. Integrated mechanical response (○——○) and per cent stimulation induced overflow (●——●) Mean ± S.E.M. of 8 experiments. The experiments illustrated in Fig. 5 were performed as control to the drug experiments shown in the following figures where drugs were present during the three last stimulations.

traction during 30 sec was due to the twitch The integrated contraction upon stimulation at 4 8 and 16 Hz was proportional to the frequency

During stimulation at 4—16 Hz a clear increase of the tritium overflow was obtained (Fig. 2) The stimulation-induced overflow (see Material and Methods) during stimulation at 16 Hz for 30 sec was 1 8 %

The fraction of the tritium content (at the onset of each stimulation) over flowing *per* impulse at different frequencies was determined by stimulation for 120 sec and was calculated to be around 3.5×10^{-5} (Fig 3) No clear differencies between the various frequencies (1—16 Hz) were obtained.

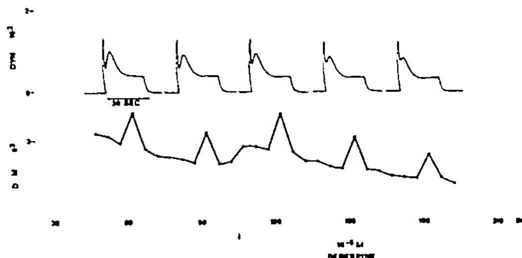


Fig 6A

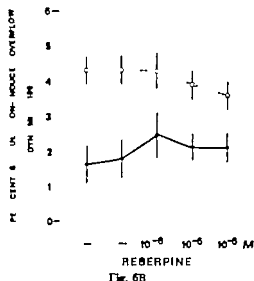
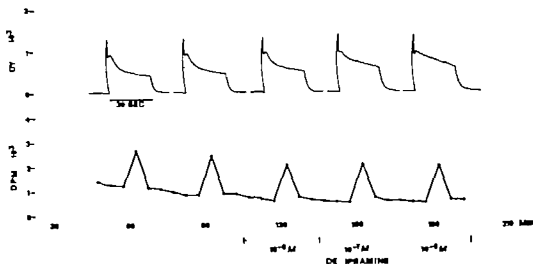


Fig. 6B

Fig 6. Stimulation of mouse vas deferens preincubated with ³H-NA. The organ was superfused with buffer and five consecutive stimulations were performed at 16 Hz for 30 sec at 30 min intervals. During the last three stimulations reserpine phosphate 10^{-6} M was added to the buffer. A. Mechanical response (upper) and tritium overflow (lower) from one experiment. B. Integrated mechanical response (○——○) and per cent stimulation-induced overflow (●——●) Mean ±S.E.M. of 4 experiments.

Effect of different stimulation time periods See Fig 4 The contractile force declined upon prolonged stimulation at 16 Hz. Repeated twitches could then often be observed. The stimulation-induced overflow was found to be proportional to the length of stimulation.

Determination of ³H-NA and its ³H metabolites See Table 1 Only about one third of the spontaneous tritium overflow was recovered as ³H NA On the other hand, upon stimulation 73 % of the tritium overflow was recovered as ³H NA. Almost all the tritium in the vas deferens at the end of superfusion was due to ³H NA


Fig. 7A

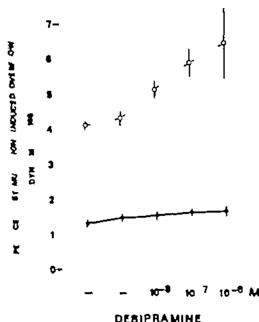


Fig 7B

Fig 7 Stimulation of a mouse vas deferens preincubated with ^{3}H NA. The organ was superfused with buffer and five consecutive stimulations were performed at 16 Hz for 30 sec at 30 min intervals. During the last three stimulations desipramine 10^{-8} M-10^{-6} M was added to the buffer A. Mechanical response (upper) and tritium overflow (lower) from one experiment. B. Integrated mechanical response (o——o) and per cent stimulation-induced overflow (●——●) Mean ±S.E.M. of 8 experiments

Drug effects

Effect of repeated stimulations See Fig 5 As control for drug experiments five consecutive stimulations were made (16 Hz, 30 sec every 30 min) during superfusion with drug-free buffer Both the mechanical activity and the stimulation-induced overflow remained fairly constant during the five stimulations.

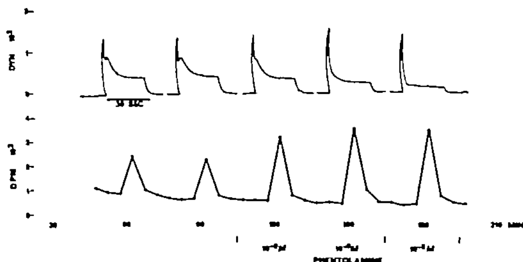


Fig. 8A

Fig. 8. Stimulation of mouse vas deferens preincubated with ^{3}H-NA. The organ was superfused with buffer and five consecutive stimulations were performed at 16 Hz for 30 sec at 30 min intervals. During the last three stimulations phentolamine 10^{-8} M – 10^{-6} M was added to the buffer. A. Mechanical response (upper) and tritium overflow (lower) from one experiment. B. Integrated mechanical response (○——○) and per cent stimulation-induced overflow (●——●). Mean ±S.E.M. of 6 experiments.

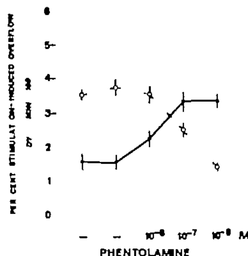


Fig. 8B

6-OH-dopamine. No contractions could be elicited in the vas deferens from mice given 6-OH-dopamine 250 mg/kg i.v. 20 h before death (4 experiments).

Atropine and propranolol. The contraction caused by stimulation was not affected by atropine 10^{-6} M (4 experiments) or propranolol 10^{-6} M (4 experiments).

Tetrodotoxin. When tetrodotoxin 3×10^{-7} M was added to the superfusing buffer no mechanical response or increased tritium overflow was obtained upon stimulation. After 30 min washing with fresh buffer both the stimulation-induced overflow of tritium and the mechanical response were almost completely restored to the control levels (4 experiments).

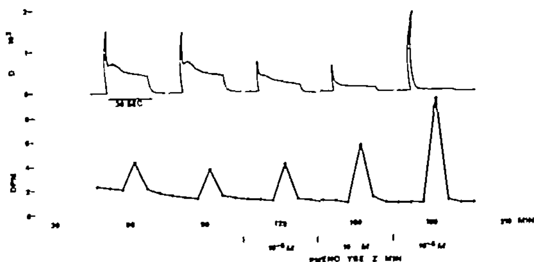


Fig. 9 A

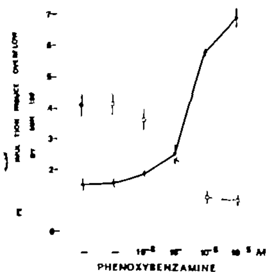


Fig. 9B

Fig 9 Stimulation of a mouse vas deferens preincubated with H NA. The organ was superfused with buffer and five consecutive stimulations were performed t 16 Hz for 30 sec at 30 min intervals. During the last three timulations phenoxybenzamine 10^{-8} M $- 10^{-5}$ M was added to the buffer A. Mechanical response (upper) and tritium over flow (lower) from one experiment. B. Integrated mechanical response (o o) and per cent stimulation-induced overflow (●——●) I this figure h bo shown a sixth experiment with phenoxybenzamine 10^{-8} M. Mean ± S.E.M. of 5 experiments.

Reserpine See Fig 6. Reserpine phosphate 10^{-6} M did not greatly affect the contraction upon stimulation. Only a slight decrease of the contraction was found after exposition to reserpine for about 1 h The spontaneous overflow of tritium was increased by reserpine Also the stimulation-indu ed overflow was lightly increased

Desipramine See Fig 7 Desipramine caused a dose-dependent potentiation of the second phase of contraction at 10 M – 10^{-6} M At 10 M the mechanical response was greatly impaired. No safe increase of th stim lation-induced overflow was obtained at any frequency tested (4 8 and 16 Hz)

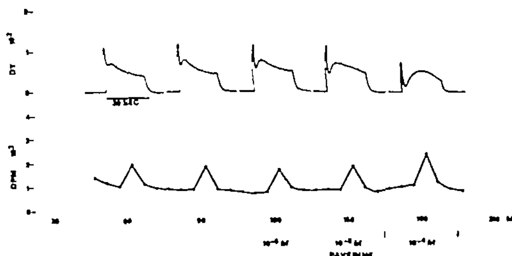


Fig 10A

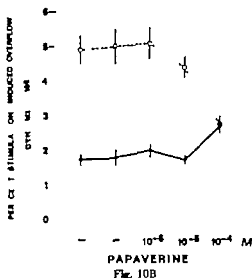


Fig 10B

Fig. 10. Stimulation of mouse vas deferens preincubated with ³H-NA. The organ was superfused with buffer and five consecutive stimulations were performed at 16 Hz for 30 sec at 30 min intervals. During the last three stimulations papaverine 10^{-6} M – 10^{-4} M was added to the buffer A. Mechanical response (upper) and tritium overflow (lower) from one experiment. B Integrated mechanical response (○——○) and per cent stimulation-induced overflow (●——●). Mean ± S.E.M. of 6 experiments.

Phentolamine See Fig 8 Phentolamine greatly reduced the second phase of contraction while the initial twitch was not clearly affected. The stimulation-induced overflow was approximately doubled by phentolamine 10^{-7} M. Similar results were obtained with azapetine phosphate.

Phenoxybenzamine See Fig 9 Phenoxybenzamine 10^{-8} M and 10^{-7} M reduced both the twitch and the second phase of contraction At phenoxybenzamine 10^{-6} M or 10^{-5} M the twitch reappeared however and was even potentiated. The second phase of contraction was almost completely abolished. Phenoxybenzamine caused a moderate (10^{-8} M – 10^{-7} M) to great (10^{-6} M or 10^{-5} M) increase of the stimulation induced overflow Similar results were obtained at 4 8 and 16 Hz.

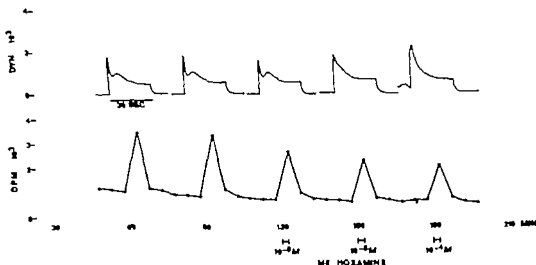


Fig. 11A

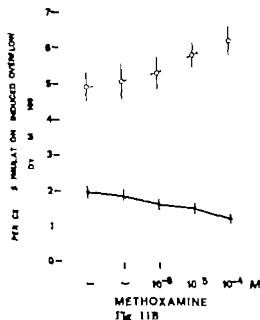


Fig 11B

Fig 11 Stimulation of a mouse vas deferens preincubated with ^{3}H NA. The organ was superfused with buffer and five consecutive stimulations were performed at 16 Hz for 30 sec at 30 min intervals. During the last three stimulations methoxamine 10^{-6} M–10^{-4} M was added to the buffer A. Mechanical response (upper) and tritium overflow (lower) from one experiment. B. Integrated mechanical response (○——○) and per cent stimulation induced overflow (●——●) Mean ± S.E.M. of 6 experiments.

Papaverine See Fig 10 Papaverine 10^{-4} M reduced the contraction to about 50 %. Both the twitch and the second phase of contraction were reduced. Papaverine 10^{-4} M slightly increased the spontaneous tritium overflow The stimulation-induced overflow was increased by about 50 %

Methoxamine See Fig 11 Methoxamine was added to the superfusing buffer for periods of 2 min starting about 1 min before stimulation Methoxamine 10^{-5} M slightly increased and methoxamine 10^{-4} M greatly increased the tension of the vas deferens. The contraction upon stimulation was increased. The stimulation-induced overflow on the other hand was decreased

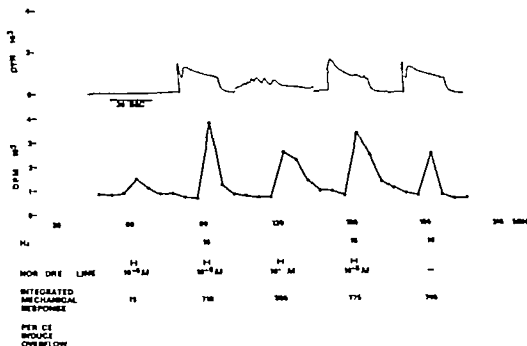


Fig. 1.. Mechanical response and tritium overflow d ring superfusion of mouse vas deferens preincubated with ^{3}H-NA. Effect of addition of exogenous NA 10^{-6} M or 10^{-5} M to the buffer and/or stimulation at 16 Hz for 30 sec as indicated in the figure Integrated mechanical response (dyn×min) nd per cent increase of tritium overflow (i.e. the increased tritium overflow caused by timulation and/or exogenous NA in per cent of the total tritium content of the vas deferens t th onset of each experiment) is given below Mean of 2 experiments.

NA See Fig 12. Exogenous NA was added to the superfusing buffer for periods of 2 min, with or without stimulation. NA 10^{-6} M did not increase the tension of the vas deferens and did not affect the contraction caused by stimulation. NA $^{-5}$ M increased the tension of the vas deferens. Stimulation did not cause any rapid twitch response, but the total contraction during stimulation was slightly increased. NA 10^{-6} M (without stimulation) slightly increased the spontaneous tritium overflow Upon stimulation, the additional (stimulation-induced) tritium overflow was not greatly affected by the presence of NA 10^{-6} M. NA 10^{-5} M markedly increased the spontaneous tritium overflow and only a very small additional increase of the over flow was caused by stimulation.

Discussion

The isolated vas deferens preparation has been widely used for studies on autonomic neuro-muscular transmission (see Holman, 1970) Contractions can be elicited in the organ either by activation of the preganglionic nerve fibers (Huković, 1961) or



Fig. 11A

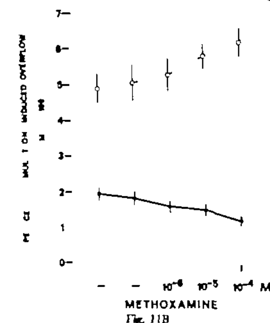


Fig. 11B

Fig. 11. Stimulation of a mouse vas deferens preincubated with ^{3}H NA. The organ was superfused with buffer and five consecutive stimulations were performed at 15 Hz for 30 sec at 30 min intervals. During the last three stimulations methoxamine 10^{-6} M–10^{-4} M was added to the buffer. A. Mechanical response (upper) and tritium overflow (lower) from one experiment. B. Integrated mechanical response (○——○) and per cent stimulation-induced overflow (●——). Mean ± S.E.M. of 6 experiments.

Papaverine. See Fig. 10. Papaverine 10^{-4} M reduced the contraction to about 50 %. Both the twitch and the second phase of contraction were reduced. Papaverine 10^{-4} M slightly increased the spontaneous tritium overflow. The stimulation-induced overflow was increased by about 50 %.

Methoxamine. See Fig. 11. Methoxamine was added to the superfusing buffer for periods of 2 min, starting about 1 min before stimulation. Methoxamine 10^{-5} M slightly increased and methoxamine 10^{-4} M greatly increased the tension of the vas deferens. The contraction upon stimulation was increased. The stimulation-induced overflow on the other hand was decreased.

deferens are graded and not all or none) decrease in amplitude (Furness personal communication)

Release of NA from adrenergic nerves can be monitored by determination of outflow of radioactively labelled NA previously taken up into the nerve terminals (Hertting and Axelrod 1961 Rosell *et al.* 1963) Almost all the radioactivity in a vas deferens, which has been preincubated with ³H NA and then superfused by fresh buffer is unchanged ³H NA, and the greatest part of the radioactivity appearing in the superfusate upon stimulation is unchanged ³H NA Thus, total radioactivity can in the present investigation be taken as an appropriate indicator of ³H NA About 2 % of the total NA content in the vas deferens is ³H NA. No change of the stimulation induced overflow occurs during superfusion for 200 min indicating that NA is not greatly redistributed during the experiment.

The fractional tritium overflow *per* impulse from the mouse vas deferens is almost the same within a wide range of frequencies (1—16 Hz) It is of the same order of magnitude as that from the isolated rat iris (Farnebo and Hamberger 1970a) and that from the isolated rat portal vein (Häggendal *et al* 1970) It is possible that different degrees of reuptake of released transmitter at different frequencies might conceal a difference in release *per* impulse (*cf* Häggendal and Malmfors, 1969) This seems unlikely however as no differences between various frequencies were found when NA uptake was inhibited by desipramine The transmurally stimulated superfused mouse vas deferens seems to be a suitable sympathetic adrenergic neuro-effector system for studies on the correlation between transmitter release and effector response.

Reserpine inhibits the Mg ATP dependent uptake mechanism of the amine storage granules (see Carlsson 1966) The increased spontaneous tritium overflow is probably mainly due to deaminated metabolites of ³H NA (Carlsson, 1960) as is the case in the isolated rat iris (see Farnebo, 1971) Reserpine *in vitro* does not greatly affect the stimulated mouse vas deferens, although the contraction was slightly decreased and the stimulation-induced tritium overflow slightly increased. This is consistent with earlier findings *in vivo* (Rosell and Sedvall, 1962 Sedvall, 1964 Malmfors, 1969) and *in vitro* (Farnebo and Hamberger 1970b) that reserpine does not inhibit the neuro-muscular transmission until the NA stores have been exhausted.

Inhibition of reuptake of released NA by desipramine does not give any safe increase of the stimulation-induced overflow of tritium. The unchanged tritium *overflow* may be due to a decreased *releas* of ³H NA from the nerve terminals (see further discussion below) Yet, this decreased release after desipramine causes a potentiated mechanical response, probably because a proportionally higher concentration of the NA released reaches the receptors when reuptake is inhibited.

The α-receptor blocking drugs phentolamine and phenoxybenzamine both reduce the second phase of contraction. The decreased contraction is accompanied by an increased stimulation-induced transmitter overflow (Brown and Gillespie, 1957) This increased overflow occurs also at concentrations of the drugs, which do not

HEDQVIST P., Studies on the effect of prostaglandins E and E₂ on the sympathetic neuromuscular transmission in some animal tissues. *Acta physiol. scand* 1970 Suppl. 345 1—40.
HERTTING, G. and J AXELROD, Fate of tritiated noradrenaline at the sympathetic nerve-endings. *Nat e* 1961 *192* 172—173
HOLMAN, M. E., Introduction to electrophysiology of ʼsceral smooth muscle. *Handbook of Physiology* 1968. Sect. 6 Vol. IV pp. 1665—1708
HOLMAN M. E., Junction potentials in smooth muscle. In *Smooth mus le* (eds. E. Bülbring, A. F Brading, A. W Jones and T Tomita) pp 244—288. London: Arnold. 1970.
HUKOVIĆ, S Responses of the isolated sympathetic nerve-ductus deferens preparation of the guine-pig. *Brit J Pharmacol.* 1961 *16* 188—194
HUKOVIĆ, S and E. MUSCHOLL, Die Noradrenalin-Abgabe aus dem isolierten Kaninchenherzen bei sympathischer Nervenreizung und ihre pharmakologische Beeinflussung. *Naunyn S hmiedeberg' A h. exp Path Pharmak* 1962. *244* 81—96
KAO C. Y., Tetrodotoxin, saxitoxin and their significance in the study of excitation phe nomena. *Pharmacol. Rev* 1966. *18* 997—1049
LA E, B. P and J A. G RHODES, Cellular interrelationships and electrical activity in tw types of smooth muscle. *J Ult astruct Res.* 1964 *10* 470—488.
MALMFORS, T., Histochemical studies on the effect of nerve impulses on exogenous catecholamines taken up into the adrenergic nerves of reserpine-pretreated animals. *Pharma ology* 1969 193—208.
MALMFORS, T and CH. SACHS, Degeneration of adrenergic nerves produced by 6-hydroxy dopamine. *Europ J Pharmacol* 1968. *3* 89—92
NORBERG K. A. and B. HAMBERGER, Th sympathetic adrenergic neuron *A t physiol. scand* 1964 *63* Suppl. 238
OHLIN, P and B. C. R. STRÖMBLAD Observations on the isolated vas deferens. *Brit. J Phar macol.* 1963 *20* 299—306
ROSELL, S I J KOPIN and J AXELROD, F t of H³-noradrenaline in skeletal muscle before and following sympathetic stimulation. *Amer J Physiol.* 1963 *205* 317—321
ROSELL, S and G SEDVALL, The rate of disappearance of vasoconstrictor responses to sympathetic chain timulation after reserpine treatment. *A t physiol. cand* 1962. *56* 306—314
SEDVALL G Short term effects of reserpine on noradrenaline levels in skeletal muscle. *Acta physiol. scand* 1964 *62* 101—108
SJÖSTRAND N O Effect of reserpine and hypogastric denervation on the noradrenaline content of the as deferens and the seminal vesicle of the guinea-pig. *Act physiol. scand* 1962. *56* 376—380
SJÖSTRAND, N O The adrenergic innervation of the vas deferens and the accessory male genital glands. *Act physiol sc d.* 1965 *65* Suppl. 257 1—82.
SJÖSTRAND, N O and G SWEDIN Potentiation by smooth muscle stimulants of the hypogastric nerv vas deferens preparation from normal and castrated guinea-pigs. *Acta physiol. scand.* 1968. *74* 47 —479
SWEDIN G Biphasic mechanical response of the isolated vas deferens to nerv stimulation. *A ta physiol scand* 1971a. *81* 574—576.
SWEDIN G Studies on neurotransmission mechanisms in the rat and guinea-pig vas deferens. *Act physiol scand* 1971b. Suppl. 369 1—34
THOENEN H. A HÜRLIMANN and W HAEFELY The effect of sympathetic nerve stimulation on olume, vascular resistance, and norepinephrine output in th isolated, perfused spleen of the cat, and its modification by cocaine *J Pharm ol p Ther* 1964a. *143* 57—63
THOENEN H A HÜRLIMANN and W HAEFELY Mode of action of imipramine and 5-(3 methylaminopropyliden)-dibenzo/a,e/cyclohepta/1,3,5/trien hydrochloride (Ro4-6011) a new antidepressant drug, on peripheral adrenergic mechanisms. *J Pharmacol. xp Ther* 1964b. *144* 405—414
THOENEN, H., A. HÜRLIMANN and W. HAEFELY Wirkungen von Phenoxybenzamin, Phentolamin und Azapetin auf adrenergische Synapsen der Katzen-milz. *Helv Physiol Pharmacol. A ta* 1964c. 22. 148—161
TOMITA, T Current spread in the smooth muscle of the guinea-pig as deferens. *J Physiol.* (Lond.) 1967 *189* 163—176.
TRANZER, J P and H. THOENEN Ultramorphologische Veränderungen der sympathischen Nervenendigungen der Katze nach Vorbehandlung mit 5- und 6-H droxy-Dopamin. *Naunyn-S hmied berg A h exp P th. Ph rmak* 196 57 343—344
TRENDELENBURG, U The supersensitivity caused by cocaine *J Pharmacol p Ther* 1959 *125* 55—65
TRENDELENBURG U R. A MAXWELL and S PL CHINO M thoxamine as tool t assess the importance of intraneuronal uptake of l-norepinephrine in th ca nictitating membrane. *J Pharm ol. exp Ther* 1970 *172* 91—99

Acta physiol scand 1971 Suppl 371 19—27

From the Department of Histology Karolinska Institutet, S-104 01 Stockholm, Sweden

Release of Monoamines Evoked by Field Stimulation— Studies on Some Ionic and Metabolic Requirements

By

LARS-OVE FARNEBO

Abstract

FARNEBO L.-O *Release of monoamines evoked by field stimulation — Studies on some ionic and metabolic requirements.* Acta physiol. scand. 1971 Suppl. 371 19—27

Electrical field stimulation of isolated tissues can cause release of ³H-monoamines previously taken up into monoamine nerve terminals. The stimulation-induced overflow of tritium was investigated in isolated irides previously incubated with ³H noradrenaline cerebral cortex slices incubated with ³H-noradrenaline or ³H-5-hydroxytryptamine and neostriatal slices incubated with ³H-dopamine. No stimulation induced tritium overflow was obtained if Ca^{++} was excluded from the buffer or tetrodotoxin was added to the buffer Mg^{++} 20 mM completely inhibited the stimulation-induced overflow from isolated rat iris. Exclusion of K^{+} slightly increased the stimulation-induced overflow At K^{+} 20 mM no stimulation-induced overflow was obtained. K^{+} 50 mM evoked a great increase of the tritium overflow Low Na^{+} concentrations reduced the stimulation-induced overflow In agreement with previous investigations it is concluded that the present stimulation technique may cause release of monoamines via action potentials propagated along the nerves.

Inhibition of oxidative phosphorylation moderately increased the stimulation-induced overflow from the rat iris. This is probably due to partial inhibition of noradrenaline reuptake into the nerve terminals. Exclusion of glucose from the buffer greatly increased both the spontaneous and the stimulation-induced tritium overflow The stimulation-induced overflow in the absence of glucose was only partially Ca^{++}-dependent. It is concluded that the release process for noradrenaline is not immediately inhibited after interference with energy-yielding processes.

Introduction

Electrical stimulation of adrenergic nerves can initiate action potentials which are conducted along the axons to the nerve terminals. Depolarization of the nerve terminals causes release of noradrenaline which then either overflows into the circulation or is taken up again into the nerve terminals. The exact mechanism for the release process is not known. Most NA[1] in the nerve terminals is stored in the amine storage granules (see Carlsson, 1966) and can be released only from these amine storage granules upon electrical stimulation (Malmfors, 1965 Almgren and Lundborg, 1970 Farnebo 1971a) Presence of extracellular Ca^{++} is essential for NA release (see review by Rubin, 1970) No other ions are obligate for transmitter release if depolarization of the nerve terminals can be accomplished (Katz and Miledi, 1967a,

[1] Abbreviations used NA = noradrenaline DA = dopamine 5-HT = 5-hydroxytryptamine.

Kirpekar and Wakade, 1968) On the other hand a certain ionic environment is necessary for NA uptake into the nerve terminals (Dengler *et al* 1962 Hamberger 1967 Bogdanski and Brodie, 1969) Furthermore, NA uptake requires metabolic energy which does not seem to be the case for NA release (Kirpekar *et al.*, 1970) By means of electrical field stimulation transmitter release from monoamine nerves can be studied both in the peripheral and the central nervous system *in vitro* (Baldessarini and Kopin, 1967 Chase *et al* 1969 Katz and Kopin 1969 Farnebo and Hamberger 1970 Farnebo, 1971 b) The aim of the present investigation was to study some ionic and metabolic requirements for transmitter release during electrical field stimulation.

Material and Methods

Adult femal albino rats (Sprague-D wley 180—200 g) were used. The animals were bled but under ether anesthesia and the eyes and the brain dissected out. Isolated irides (with the ciliary bodies tached) were prepared (Malmfors, 1965) and immediately immersed in cool buffer After chilling the brain in buffer f r few min tes, round slices (diameter 3 mm, weight about 3 mg) of cerebral cortex and neostriatum were prepared by means of razor blade, a frosted object slide and a cylindrical punch (McIlwain and Rodnight, 196 ; Hamberger 1967) Isolated irides were incubated with ^{3}H NA 10 M, cerebral cortex slices incubated with ^{3}H-NA or ^{3}H 5-HT 10^{-7} M and neostriatal slices incubated with H DA 10 M for 30 min at 37 C in a Krebs-Ringer bicarbonat buffer (composition in mmoles per liter NaCl, 118 KCl, 4.8 $CaCl_2$, 1.3 KH_2PO 1.2 MgSO 1 2 $NaHCO_3$ 25 ascorbic acid, 1 1 glucose, 10) equilibrated with 6 5 % CO_2 in O_2 The tissue was after rapid rinsing in buffer transferred to small chambers where it was superfused by buffer t 37 C (0.5 ml/min) In some experiments the composition of the superfusing buffer was modified. Small changes of the osmotic pressure, caused by exclusion of Ca^{++} K or glucose were not compensated for When the concentration of Ca^{++} Mg^{++} or K was increased this was compensated for by decreasing the Na concentration. When low N concentrations were investigated, osmotic pressure was kept constant by the addition of sucrose I some experiments the superfusing buffer was saturated with 6.5 % CO_2 in N instead of O_2.

After superfusion for 30 min the tissue was stimulated by an electrical field generated between platinum electrodes at the two ends of the chamber (biphasic pulses 2 msec, 1 mA, 10 Hz see Farnebo and Hamberger 1970) The irides were stim lated for 10 min and f rther super fused for 15 min, wh le the brain slices were stimulated for 2 min and further superfused for 15 min. The superfusate was collected in 5 min fractions directly in vials for liquid scintillation counting Total radioactivity in the uperfusat was determined after addition of 5 ml Insta-Gel® by counting in Packard Model 3320 Spectrometer Total radioactivity in the tissue at the end of the superfusion was determined after solubilization of the tissue in Soluene® and addition of 10 ml toluene scintillation solution.

The tritium overflow (in dpm) caused by timulation was calculated by subtracting the *estimated spontaneous tritium* overflow from the total tritium overflow during timulation. Stimulation-induced overflow was als expressed as pe cent of the total tissue tritium at the onset of timulation (see F rnebo and Hamberger 1971)

Substan *d dl*-noradrenaline-7 ^{3}H HCl (5—10 Ci/mmole, Radiochemical Centre, Amersham) dopamine-7 H HB 5-hydroxytryptamine-^{3}H(G)-creatinine sulphat (5—10 Ci/mmole, New England Nuclear Corp., Boston) 2,4-dinitrophenol (Merck) ouabaine (g-strophantin, Sandoz) tetrodotoxi (Sankyo, Japan)

Results

Isolated irides and cerebral cortex slices incubated with H NA were used for studies on release from NA nerve terminals whil cerebral cortex slices and neostriatal slices incubated with ^{3}H 5-HT and ^{3}H DA, respecti el were used for studies on release from 5-HT and DA nerve terminals (Farnebo 1971b) After in-

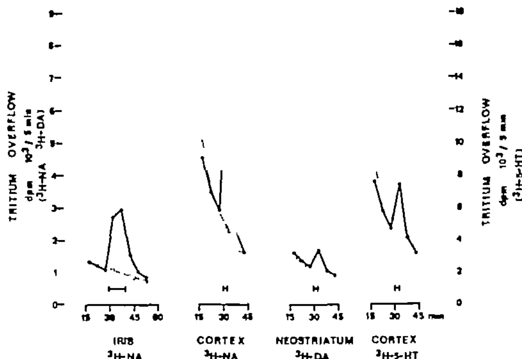


Fig 1 Tritium overflow into the buffer during superfusion and electrical field stimulation of isolated tissues. Isolated irides were incubated with ^{3}H NA 10^{-7} M, cerebral cortex slices with ^{3}H-NA 10^{-7} M or ^{3}H 5-HT 10^{-7} M and neostriatal slices with ^{3}H DA 10^{-7} M for 30 min. The tissue was then superfused by ordinary Krebs-Ringer bicarbonate buffer (●—●) Ca^{++}-free buffer (O O) or buffer containing tetrodotoxin 3×10^{-7} M (□ □) After superfusion for 30 min the tissue was stimulated by an electrical field for 10 min (iris) or 2 min (brain slices) (|—|) and then further superfused for 15 or 13 min, respectively The tritium overflow per 5 min fraction is presented. The mean tritium content of the tissue at the onset of stimulation was about 40 000 dpm in the irides and the slices incubated with ^{3}H NA or ^{3}H DA, and about 80 000 dpm in the slices incubated with ^{3}H 5-HT Mean of 6—8 experiments.

cubation with tritiated amine the tissue was superfused with normal Krebs-Ringer bicarbonate buffer for 30 min before it was stimulated by an electrical field. Stimulation increased the tritium overflow from all tissues (Fig 1)

Effect of tetrodotoxin and changes in the ionic composition of the buffer

Addition of tetrodotoxin to the superfusing buffer or exclusion of Ca^{++} from the buffer during incubation and superfusion completely inhibited the stimulation-induced increase of the tritium overflow in all tissues studied (Fig 1) The stimulation-induced tritium overflow from isolated irides was increased, when the Ca^{++} concentration was increased from 1.3 mM to 5 mM (Table 1) A high concentration of Mg^{++} (20 mM) completely inhibited stimulation-induced overflow Exclusion of K^+ from the buffer produced a small increase of the stimulation-induced overflow from isolated irides. No effect of stimulation was obtained when the K^+ concentration was increased to 20 mM (Fig 2) K^+ 50 mM evoked a marked increase of the tritium

Kirpekar and Wakade 1968) On the other hand, a certain ionic environment is necessary for NA uptake into the nerve terminals (Dengler *et al.*, 1962 Hamberger 1967 Bogdanski and Brodie 1969) Furthermore, NA uptake requires metabolic energy which does not seem to be the case for NA release (Kirpekar *et al.*, 1970) By means of electrical field stimulation transmitter release from monoamine nerves can be studied both in the peripheral and the central nervous system *in vitro* (Baldessarini and Kopin, 1967 Chase *et al* 1969 Katz and Kopin 1969 Farnebo and Hamberger 1970 Farnebo, 1971 b) The aim of the present investigation was to study some ionic and metabolic requirements for transmitter release during electrical field stimulation.

Material and Methods

Adult female albino rats (Sprague-Dawley 180—200 g) were used. The animals were bled but under ether anesthesia and the eyes and the brain dissected out. Isolated irides (with the ciliary bodies attached) were prepared (Malmfors 1965) and immediately immersed in cool buffer. After chilling the brain in buffer for few min ten, round slices (diameter 3 mm, weight about 5 mg) of cerebral cortex and neostriatum were prepared by means of a razor blade a frosted object slide and a cylindrical punch (McIlwain and Rodnight, 1962 Hamberger 1967) Isolated irides were incubated with ^{3}H NA 10^{-7} M cerebral cortex slices incubated with ^{3}H-NA or ^{3}H 5-HT 10^{-7} M and neostriatal slices incubated with ^{3}H-DA 10^{-7} M for 30 min at 37 C in Krebs-Ringer bicarbonate buffer (composition in mmoles per liter: NaCl, 118 KCl, 4.8 $CaCl_2$, 1.3 KH_2PO_4 1.2 $MgSO_4$ 1.2 $NaHCO_3$, 25 ascorbic acid, 1.1 glucose 10) equilibrated with 6.5 % CO_2 in O_2. The tissue was after rapid rinsing in buffer transferred to small chambers where it was perfused by buffer at 37 C (0.5 ml/min) In some experiments the composition of the superfusing buffer was modified. Small changes of the osmotic pressure caused by exclusion of Ca^{++} K^+ or glucose were not compensated for. When the concentration of Ca^{++} Mg^{++} or K^+ was increased, this was compensated for by decreasing the Na^+ concentration. When low Na^+ concentrations were investigated osmotic pressure was kept constant by the addition of sucrose. In some experiments the superfusing buffer was saturated with 6.5 % CO_2 in N_2 instead of O_2.

After superfusion for 30 min the tissue was stimulated by an electrical field generated between platinum electrodes at the two ends of the chamber (biphasic pulses, 2 msec, 12 mA 10 Hz see Farnebo and Hamberger 1970) The irides were stimulated for 10 min and further superfused for 15 min, while the brain slices were stimulated for min and further superfused for 15 min. The superfusate was collected in 5 min fractions directly in vials for liquid scintillation counting. Total radioactivity in the perfusate was determined after addition of 5 ml Insta-Gel® by counting in a Packard Model 3320 Spectrometer. Total radioactivity in the tissue at the end of the superfusion was determined after solubilization of the tissue in Soluene® and addition of 10 ml toluene scintillation solution.

The tritium overflow (in dpm) caused by stimulation was calculated by subtracting the estimated spontaneous tritium overflow from the total tritium overflow during stimulation. Stimulation-induced overflow was also expressed as per cent of the total tissue tritium at the onset of stimulation (see Farnebo and Hamberger 1971)

Substances used *dl*-noradrenaline-7 ^{3}H HCl (5—10 Ci/mmole Radiochemical Centre Amersham), dopamine 7 ^{3}H HBr 5-hydroxytryptamine-^{3}H(G)-creatinine sulphate (5—10 Ci/mmole, New England Nuclear Corp Boston) 2,4-dinitrophenol (Merck) ouabaine (g-strophantin, Sandoz) tetrodotoxin (Sankyo, Japan)

Results

Isolated irides and cerebral cortex slices incubated with ^{3}H NA were used for studies on release from NA nerve terminals, while cerebral cortex slices and neostriatal slices incubated with ^{3}H 5-HT and ^{3}H DA, respectively were used for studies on release from 5-HT and DA nerve terminals (Farnebo, 1971b) After in

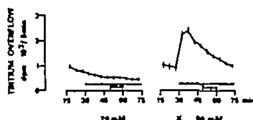


Fig. 2. Tritium overflow into the buffer during superfusion and stimulation of isolated irides previously incubated with ³H-NA 10^{-7} M. The irides were superfused by ordinary Krebs-Ringer bicarbonate buffer for 30 min before the concentration of K was increased to 70 mM or 50 mM (□). After superfusion with high K concentration for 20 min the irides were stimulated by an electrical field for 10 min (├─┤) and further superfused for 15 min. The tritium overflow per 5 ml fraction is presented. Mean ± S.E.M. of 4—8 experiments.

overflow and no further increase was caused by electrical stimulation. The stimulation-induced overflow was considerably decreased, when the Na concentration was reduced and Na replaced by sucrose. Addition of Li 2.4 mM did not affect the stimulation-induced overflow.

Effect of anoxia, exclusion of glucose and metabolic inhibitors

When the superfusing buffer was equilibrated with N_2/CO_2 a Ca^{++}-dependent, slightly increased stimulation-induced overflow was obtained (Table 2). A slight increase of the stimulation induced overflow was also obtained when NaCN or 2,4-dinitrophenol was added to the buffer. Exclusion of glucose markedly increased the spontaneous tritium overflow (Fig. 3) regardless of equilibration of the buffer with O_2/CO_2 or N_2/CO_2. Electrical stimulation greatly increased the tritium overflow (Table 2). The stimulation induced overflow in the absence of glucose was only

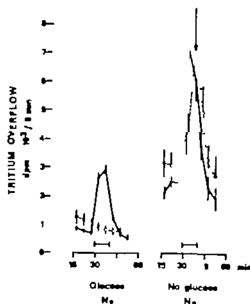
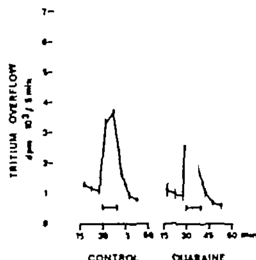


Fig. 3. Tritium overflow into the buffer during superfusion and stimulation of isolated irides previously incubated in oxygenated Krebs-Ringer bicarbonate buffer containing ³H-NA 10^{-7} M. The irides were superfused by buffer equilibrated with N_2/CO_2 with or without glucose. The Ca^{++} concentration was either 1.5 mM (●—●) or zero (O—O) and in the latter case the incubation was also performed in Ca^{++}-free buffer. After superfusion for 30 min the irides were stimulated by an electrical field for 10 min (├─┤) and further superfused for 15 min. The tritium overflow per 5 min fraction is presented. Mean ± S.E.M. of 8—14 experiments.

Fig 4 Tritium overflow into the buffer during superfusion and stimulation of isolated irides previously incubated with ^{3}H NA 10^{-7} M The irides were superfused by drug free buffer or buffer containing ouabaine 10^{-4} M for 30 min. The irides were then stimulated by an electrical field for 10 min (⊢⊣) and further superfused for 15 min. The tritium overflow per 5 min fraction is presented. Mean ± S.E.M of 8 experiments



partially Ca -dependent. Ouabaine greatly increased the tritium overflow during the first 5 min period of stimulation, while the overflow during the second 5 min period was far below the control (Fig 4)

Discussion

Electrical field stimulation causes an increased transmitter overflow from adrenergic nerve terminals as well as from central NA DA and 5-HT nerve terminal (Baldessarini and Kopin 1967 Chase *et al* 1969 Katz and Kopin 1969 Farnebo and Hamberger 1970 Farnebo 1971 a, b) The increased tritium overflow caused by the present stimulation technique can be completely inhibited by the addition of tetrodotoxin to the superfusing buffer On the other hand, field stimulation by *high* current can cause transmitter overflow which is not affected by tetrodotoxin (Katz and Kopin, 1969) Tetrodotoxin inhibits conduction of action potentials along nerves by preventing the regenerative influx of Na through the nerve cell membrane (see Kao, 1966) but does not inhibit transmitter release caused by local depolarization of the nerve cell membrane (Katz and Miledi, 1967 a, b) Thus, monoamine release caused by the present stimulation technique seems to be mediated via action potentials, initiated by the stimulation and propagated along the nerves.

Removal of Ca does not affect propagation of action potentials along nerves, but completely abolishes transmitter release from cholinergic (Katz and Miledi, 1965) and adrenergic nerves (Boullin 1967 Kirpekar and Misu, 1967) No stimulation-induced overflow is obtained during field stimulation, if Ca is excluded from the buffer (*cf* Katz and Kopin, 1969) Increased concentrations of Mg^{++} which may compete with Ca for common sites on the membrane (see Rubin, 1970) almost completely suppress stimulation-induced overflow (Boullin, 1967 Kirpekar and Misu, 1967)

Removal of K from the superfusing buffer slightly increases the stimulation-induced overflow from isolated rat iris. As K is necessary for reuptake of NA into the nerve terminals this may be the reason for the increased overflow A possible mechanism may also be an increased release from the nerve terminals. Addition of K 20 mM to the superfusing buffer has no effect on the spontaneous tritium overflow and no stimulation-induced overflow is obtained (Fig 2) It is probable that the increased extracellular K concentration causes a depolarization of the nerve cell membrane which is sufficient to cause blockade of nerve impulse conduction, but is not sufficient to cause transmitter release (*cf* Katz and Miledi 1967 b) On the other hand, K 50 mM causes a depolarization of the nerves, which is large enough to evoke a pronounced transmitter release

The reduced stimulation-induced overflow at low Na is probably due to blockade of nerve impulse conduction and not due to influence on the release process proper as previously shown in experiments where depolarization was induced by high K (Kirpekar and Wakade, 1968) or by local stimulation (Katz and Miledi, 1967 a)

Addition of Li 2.4 mM to the buffer did not affect the stimulation-induced overflow (*cf* Katz *et al.*, 1968 Bindler *et al.* 1971)

NA uptake into the adrenergic nerves seems to be linked to a Na—K activated ATP-ase and ouabaine inhibits this transport enzyme as well as NA uptake (Dengler *et al.*, 1962 Hamberger 1967 Bogdanski and Brodie 1969) In the present experiments ouabaine produced a pronounced increase of the stimulation-induced overflow during the first 5 min period of stimulation, which may be due to inhibition of NA uptake. The low stimulation-induced overflow during the second 5 min period of stimulation is probably due to progressive depletion of intracellular K from the very fine nerve fibers, which leads to decreasing amplitude of the action potentials and finally block of nerve impulse conduction (see Katz, 1966 Katz and Kopin 1969)

Inhibition of oxidative phosphorylation by anoxia, NaCN or 2,4-dinitrophenol does not inhibit, but rather increases the stimulation induced overflow from isolated rat iris. Uptake of NA into the adrenergic nerves requires energy which can be supplied either through oxidative phosphorylation or glycolysis (Dengler *et al.*, 1962 Hamberger 1967) The moderate increase of the stimulation-induced overflow after inhibition of oxidative phosphorylation is most likely due to partial inhibition of NA uptake. Efficient inhibition of NA uptake by desipramine increases stimulation-induced overflow in the isolated rat iris to about 20 % (Farnebo and Hamberger 1971) Similar results have been obtained by Kirpekar *et al.* (1970) although these authors did use phenoxybenzamine in their experiments which does not make the results fully comparable.

Glucose deprivation causes a great increase of both the spontaneous and the stimulation-induced tritium overflow These effects can only to a certain extent be explained by inhibition of NA uptake as potent inhibitors of NA uptake do not increase spontaneous NA release and only give a moderate increase of the stimulation induced overflow (Hamberger 1967 Farnebo and Hamberger 1971) It thus seems

probable that an intact inward and/or outward transport system for NA at the level of the cell membrane is not a prerequisite for transmitter release

The stimulation-induced overflow is only partially dependent on the Ca concentration after inhibition of oxidative phosphorylation and glycolysis. The release in this case thus differs from the normal. However most functions in the nerve cell are certainly impaired by this treatment *e.g* the Na—K transport in the cell membrane and the storage of NA in the amine storage granules which initially can lead to an increased release but during prolonged treatment prevents both storage and release of NA (Kirpekar *et al.*, 1970) Thus the release process for NA is not immediately inhibited after interference with energy yielding processes.

Acknowledgement

This investigation has been supported by a research grant from the Swedish Medical Research Council (B72 14X 2330-03A) The skillful technical assistance of Miss Ulla Enberg is gratefully acknowledged

References

ALMGREN O and P G LUNDBORG Release of ^{3}H metaraminol by adrenergic nerve stimulation in reserpine-treated rats. *Europ J Pharmacol.* 1970 *9* 341—346.

BALDESSARINI, R. J and I J KOPIN The effect of drugs on the release of norepinephrine-H^{3} from central nervous system tissues by electrical stimulation *in vitro. J Pharmacol. exp Ther* 1967 *156* 31—38.

BRADLER, E. H M. B WALLACH and S GERSHON, Effect of lithium on the release of ^{14}C-norepinephrine by nerve stimulation from the perfused cat spleen. *Arch. int Pharmacodyn.* 1971 *190* 150—154

BOGDANSKI D F and B. B BRODIE The effects of inorganic ions on the storage and uptake of ^{3}H-norepinephrine by rat heart slices. *J Pharmacol exp Ther* 1969 *165* 181—189

BOULLIN D J The action of extracellular cations on the release of the sympathetic transmitter from peripheral nerves. *J Physiol.* (Lond.) 1967 *189* 85—99

CARLSSON A. Drugs which block the storage of 5-hydroxytryptamine and related amines. In *Handb d Exp Pharmakol* (Ed. O Eichler and A. Farah) Springer Verlag, Berlin—Heidelberg—New York 1966. XIX 529—592.

CHASE T N R I KATZ and I J KOPIN Release of ^{3}H-serotonin from brain slices. *J Neurochem* 1969 *16* 607—615

DENGLER, H. J I A. MICHAELSON, H. E. SPIEGEL and E. TITUS, The uptake of labelled norepinephrine by isolated brain and other tissues of the cat. *Int J Neuropharmacol.* 1962. *1* 23—38

FARNEBO L.-O, Effect of reserpine on release of ^{3}H-noradrenaline ^{3}H-dopamine and ^{3}H metaraminol from field stimulated rat iris. *Biochem Pharmacol.* 1971 a. *20* 715—2776.

FARNEBO L.-O Histochemical demonstration of transmitter release from noradrenaline, dopamine and 5-hydroxytryptamine nerve terminals in field stimulated rat brain slices. *Z. Zellforsch* 1971b. *122* 503—519

FARNEBO, L.-O and B. HAMBERGER, Release of norepinephrine from isolated rat iris by field stimulation *J Pharmacol exp Ther* 1970. *172* 332—341

FARNEBO L.-O and B. HAMBERGER, Drug-induced changes in the release of ^{3}H noradrenaline from field stimulated rat iris. *Brit J Pharmacol* 1971 *43* 97—106

HAMBERGER, B Reserpine-resistant uptake of catecholamines in isolated tissues of the rat. A histochemical study *Acta physiol. scand* 1967 Suppl. 295 1—56

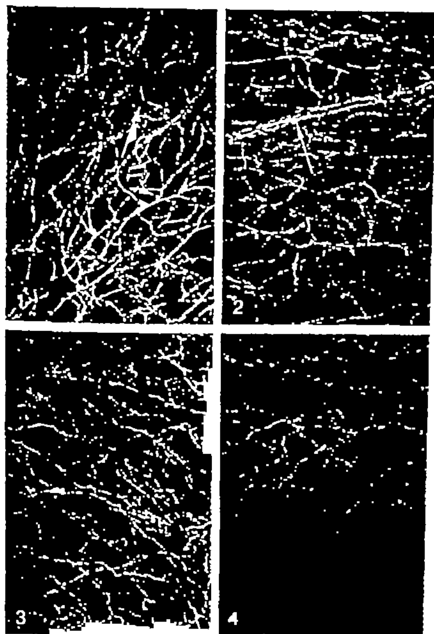
KAO C. Y, Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmacol. Rev* 1966. *18* 997—1049

KATZ, B., *Nerve muscle and synapse* McGraw-Hill, New York. 1966

KATZ, B. and R MILEDI, The effect of calcium on acetylcholine release from motor nerve terminals. *Proc Roy Soc* (Lond) ser B vol 161 pp 496—503 1965

KATZ, B. and R. MILEDI, Ionic requirements of synaptic transmitter release. *Nature* 1967a. *215* 651

Katz B and R. Miledi A tudy of synaptic transmission i the bsence of nerve impulses. *J Physiol.* 1967b. *192* 407—436
Katz, R. I T N Chase and I J Kopin Evoked release of norepinephrine and serotonin from brain slices: inhibition by lithium. *Scien e* 1968. *162* 466—467
Katz, R. L and I J Kopin Release of norepinephrine H and serotonin-³H evoked f om brai slices by lectrical field stimulation, calcium dependency and the effects of lithium ouabain and tetrodotoxin. *Biochem. Pharm ol.* 1969 *18*. 1935—1939
Kirpekar, S. M and Y Misu Release of noradrenaline by plenic nerve timulation and its dependence of calcium. *J Physiol* (Lond.) 1967 *188* 219—235
Kirpekar, S. M., J C. Pr t and H. Yamamoto Effects of metabolic hibitors on nor epinephrine release from the perfused spleen of the cat. *J Ph rma ol p Ther* 1970. *172*. 312—350.
Kirpekar, S. M and A. R. Wakade Release of noradrenaline from the t splee by potassium. *J Physiol.* 1968. *94* 595—608.
Malmfors, T Studies on adrenergic nerves. The se of rat and mouse iris for direct observa tion on their physiology and pharmacology at cell la and subcell la levels. *Acta phy iol scand.* 1965 *64* Suppl. 18. 1—93
McIlwain H and R. Rodnight *P ctic I N ochem try* J A Churchill, London, 1962.
Rubin R. P The role of calcium in the release of neurot ansm tter substances and hormones. *Pharmacol Rev* 1970 22 389—4 8.



Figs 1—4 Fluorescence micrographs of stretch-prepared rat irides treated for formaldehyde-induced fluorescence visualization of NA (× 240)

Fig. 1 Superfused control iris from H44/68 pretreated rat A dense network of intensely fluorescent, varicose nerve terminals and some weakly fluorescent, smooth non-terminal axons (→) can be seen The appearance is indistinguishable from that of superfused control iris from an untreated rat.

Figs 2 and 3 Stimulated iris from untreated (Fig 2) or FLA63 pretreated (Fig 3) rat. Slightly less prominent varicosities but more prominent non-terminal axons (→) compared to Fig. 1

Fig. 4 Stimulated iris from H44/68 pretreated rat. Most of the nerve terminals show smoother appearance and are less intensely fluorescent than those in Fig 1 A few nerve terminals seem to be more or less unaffected by stimulation

Results

Enzymatic assay of endogenous NA The NA content of irides of untreated rats, taken for assay directly after dissection, was found to be about 5 ng/iris (Table 1) Incubation for 30 min and superfusion for 60 min with buffer did not alter the NA content. Stimulation for 60 min (during superfusion) of irides from untreated or FLA63 pretreated rats lowered the NA content to about 3 ng/iris a decrease to about 60 % of the control irides Addition of L-tyrosine to the superfusing buffer did not diminish the depletion of NA caused by stimulation In irides of H44/68 pretreated rats, stimulation lowered the NA content to 1 2 ng/iris a decrease to 26 % of the control irides.

Histochemistry Incubation of irides for 30 min and superfusion for 60 min did not change the appearance of the adrenergic nerves compared to irides which were stretch-prepared directly after dissection In irides from untreated rats, stimulation for 60 min did not cause any distinct decrease of the fluorescence intensity of the majority of the adrenergic nerve terminals. In some areas the fluorescence intensity was moderately decreased and the varicosities had become slightly less prominent compared to the unstimulated control irides (Figs. 1 and 2) *On the other hand* the non-terminal axons were more prominent in the stimulated irides than in the unstimulated irides. The same difference between stimulated and unstimulated irides was found, when tyrosine was present in the superfusing buffer or when the animal had been pretreated with the dopamine-β-hydroxylase inhibitor FLA63 (Fig 3) In irides from animals pretreated with the tyrosine hydroxylase inhibitor H44/68 1 h stimulation greatly reduced the fluorescence intensity of most of the nerve terminals (Fig 4) Some nerve terminals had a very low fluorescence intensity without prominent varicosities while a few others seemed to be more or less unaffected by stimulation. The non-terminal axons were slightly more prominent than those of unstimulated irides.

Discussion

The endogenous NA content of an iris from untreated rat was found to be about 5 ng which is in good agreement with previous results obtained with a fluorimetric method (Dahlström *et al.*, 1966) Electrical field stimulation can cause release of NA from adrenergic nerves in isolated tissues (Baldessarini and Kopin, 1967 Farnebo and Hamberger 1970) Stimulation at 10 Hz for 60 min lowers the NA content to about 60 % In the fluorescence microscope an altered distribution of NA can be observed with a less pronounced cumulation of NA to the varicosities of the adrenergic nerve terminals. The increased fluorescence intensity of the non-terminal axons is probably due to uptake of NA released from the nerve terminals (Malmfors, 1965) Inhibition of dopamine β-hydroxylase by FLA63 does not cause any further depletion of NA by stimulation (*cf* Corrodi *et al.* 1970)

Addition of tyrosine to the superfusing buffer does not diminish the NA depletion caused by stimulation of irides of untreated rats. The conversion of tyrosine to

dihydroxyphenylalanine (DOPA) has been shown to be the rate limiting step in NA synthesis in the adrenergic nerves (Spector *et al* 1965) The stimulation induced decrease of NA is thus probably not due to lack of substrate for new synthesis, but rather due to insufficient capacity of the enzyme to convert tyrosine to dihydroxyphenylalanine Stimulation of irides from rats pretreated with the tyrosine hydroxylase inhibitor H44/68 1 h before killing lowers the endogenous NA content to about 25 % This decrease can be seen as a marked reduction of the fluorescence intensity in most of the adrenergic nerve terminals (Corrodi and Malmfors, 1966)

Exogenous ^{3}H NA is relased to about the same, or even to a lower extent from irides of H44/68 pretreated rats compared to irides of untreated rats (Farnebo and Hamberger 1970) The release of endogenous NA is thus probably not lower from irides of untreated rats. The difference found in stimulation-induced depletion of endogenous NA between irides of untreated and H44/68 pretreated rats is most likely due to synthesis of NA *in vitro* in the irides of untreated rats. A preferential release of newly synthesized NA (Kopin *et al* 1968 Sedvall 1969 Stjärne and Wennmalm, 1970) probably takes place also in the isolated rat iris, and the synthesis of NA may thus be even greater

Acknowledgements

This investigation has been supported by research grants from the Swedish Medical Research Council (B72 14X-2330-05A, B72 14X 2295-05A)

For generous supplies of drugs we are indebted t AB Astra (FLA63) nd AB Hässle (H44/68) through Dr H Corrod

References

Baldessarini, R. J and I J Kopin The effect of drugs on the release of norepinephrine-H^3 from central nervous system tissues by electrical stimulation *in vitro* *J Pharmacol e p Ther* 1967 *156* 31—38.

Corrodi, H., K. Fuxe, B. Hamberger and A. Ljungdahl, Studies on central and peripheral noradrenaline neurons using new dopamine-β-hydroxylase inhibitor *Europ. J Pharmacol.* 1970. *12* 145—155.

Corrodi, H. and T Malmfors, The effect of nerve activity on the depletion of the adrenergic transmitter by inhibitors of noradrenaline synthesis. *Act physiol. scand* 1966. *67* 352—357

Dahlström, A., J Häggendal and T Hökfelt The noradrenaline content of the varicosities of sympathetic adrenergic nerve terminals in the rat. *A t physiol. scand* 1966 *67* 289—294

Falck, B Observations on the possibilities for the cellular localization of monoamines with fluorescence method. *Acta physiol. scand* 1962. *56* Suppl. 197 1—25

Farnebo, L.-O and B Hamberger, Release of norepinephrine from isolated rat iris by field stimulation. *J Pharmacol. p Ther* 1970 *172* 332—341

Iversen, L. L., The uptake and torage of noradrenaline i sympathetic nerves. Cambridge University Press. 1967

Iversen L. L. and B. Jarrott Modification of an enzyme assay procedure for noradrenaline. *Biochem Pharmacol.* 1970 *19* 1841—1843

Jonsson, G Microfluorimetric studies on the formaldehyde-induced fluorescence of noradrenaline in adrenergic nerves of rat iris. *J Histochem. Cytochem* 1969 *17* 714—723

Kopin, I. J., G R. Breese, K. R. Krauss and V K. Weise, Selective release of newly synthesized norepinephrine from the cat spleen during sympathetic nerve stimulation. *J Pharmacol. p. Ther* 1968 *161* 271—287

LIDBRINK, P and G JONSSON Semiquantitative estimation of formaldehyde-induced fluorescence of noradrenaline in central noradrenaline nerve terminals. *J Histochem Cytochem.* 1971 (in press)
MALMFORS T Studies on adrenergic nerves. The use of rat and mouse iris for direct observation on their physiology and pharmacology t cellular and subcellular levels. *Acta physiol. scand* 1965 *64* Suppl. 248 1—93
SAELENS J K., M. S SCHOEN and G B KOVACSICS, An enzyme a ay for norepinephrine in brain tissue *Biochem Pharm col.* 1967 *16* 1013—1019
SEDVALL, G C., Effect of nerv timulation on accumulation and disappearance of catecholamines formed from radioactive precursors in In *M tabolism of amines in the brain.* (ed. G Hooper) pp. 23—28 Macmillan London. 1969
SPECTOR, S A. SJOERDSMA and S. UDENFRIEND, Blockade of endogenous NE synthesis by α-methyl-tyrosine an inhibitor of ty osine hydroxylase. *J Pharmacol p Ther* 1965. *147* 86—95
STJÄRNE, L. and A. WENNMALM Preferential secretion of newly formed noradrenaline in the perf sed rabbit heart. *Acta physiol. cand.* 1970 *80* 4 8—429

ably indirectly increase activation of receptors and either do not affect turnover or decrease turnover (Corrodi *et al* 1967 b Corrodi and Fuxe 1969 Meek and Werdinus 1970) However the effects of these drugs on the monoamine release in the central nervous system have not been extensively studied

In the peripheral nervous system it has been proposed that the release of NA *per* nerve impulse from the adrenergic nerve terminals is not constant but variable and depending on the active state of the effector cell (see Haggendal 1970) In agreement with this, studies on field stimulated rat iris or mouse vas deferens indicated that drugs increasing effector response decrease NA release while drugs decreasing effector response increase NA release (Farnebo and Hamberger 1970 a, 1971 Farnebo and Malmfors, 1971) These findings were taken as indications for the existence of a *local* negative feed-back mechanism for regulation of NA release in the adrenergic neuro-effector system (see also Hedqvist 1970)

Electrical field stimulation of brain slices can be used for studies on transmitter release from central monoamine nerve terminals (Baldessarini and Kopin 1967 Chase *et al.* 1969 Katz and Kopin 1969 Farnebo and Hamberger 1970 a Farnebo 1971 a) The aim of the present investigation was to study how psychoactive drugs influence this release in order to find out if a local regulation of transmitter release exists also in the central nervous system.[2]

Material and Methods

Adult female albino rats (Sprague-Dawley 180—?00 g) were used. Th animals wer bled out under ether anesthesia and the brains rapidly dissected out and cooled in Krebs-Ringer bicarbonate buffer Round lices (diameter 3 mm, weight bout 5 mg) of cerebral cortex and eostriatum were prepared by using a razor blade, a f osted object slide and a cylindrical punch (McIlwain and Rodnight, 196? Hamberger 1967) The slices were immediat ly immersed in cool buffer and then incubated for 30 min at 37 C in a Krebs-Ringer bicarbonat buffer equilib ted w th 6.5 ° CO_2 in O_2. Cerebral cortex slices were incubated with H NA 10 ⁷ M or H 5-HT 10 ⁷ M and neostriatal slices incubated with ³H DA 10 ⁷ M After rinsing for a few sec n fresh buffer the lices were transferred to small stimulation chambers where they were superfused by buffer containing the drug to be tested After superfusion for 30 min the slices were tim lated by an electrical field generated between two platinum electrodes in the chamber by Grass S-4 stimulator [biphasic pulses, 2 msec, 10 Hz, 12 mA (cerebral cortex) or 20 mA (neostriatum)] for min and then further superfused for 13 min (Farnebo and Hamberger 1970 b) The superfusate (0.5 ml/min) was collected directly in 5 min fractions in coun ng ials *and total radioactivity determined by counting in Packard 3320 Liquid* Scintillation Spectrometer after addition of 5 ml Insta-Gel® scintillation sol tion. At the end of superfusio th slices were solub lized in 0 5 ml Soluene® and radioactivity was determined after th addition of 10 ml tol ene scintillation sol tion. Quenching was determined by recounting representativ samples fter addition of a standard amount of ³H toluene The tritium overflow induced by stimulation was calculated by subtracting the estimated spontaneous tritium overflow from the total tritium overflow (see Fig 1) The stimulation-induced overflow was also expressed as per cent of the total trit um content in the tissue t the onset of stimulation.

Substanc s used *dl*-noradrenaline-7 H HCl (5—10 Ci/mmole Radiochemical Centre, Amersham) 5-hydroxytryptamine (G)³H-creatinine sulphate (5—10 Ci/mmole New England Nuclear Boston) dopamine 7 H HBr (5—10 Ci/mmole, New England Nuclear), desipramine (Pertofran® Geigy) clonidine (Catapresan® Boehringer) phenoxybenzamine (Dibenyline® Smith Kline & French) phentolamine (Regitin® Ciba) chlorpromazine (Hibernal® Leo); chlorimipramine (Geigy) D-lysergic acid diethylamid (Sandoz) cocaine HCl pomorphine (Sandoz); pimozid (Janssen Beerse) Insta-Gel® nd Soluene® (Packard)

[2] Some of the present results have previously been p blished (Farnebo and Hamberger 1970)

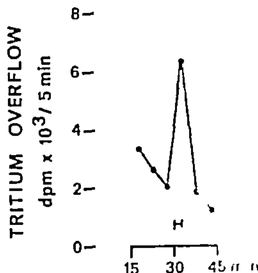


Fig. 1. Tritium overflow into the buffer during superfusion and stimulation of a cerebral cortex slice preincubated with ³H NA 10^{-7} M 30 min. After superfusion for 30 min, the slice was stimulated for 2 min (├─┤) and further superfused for 15 min before total tritium content of the slice was determined. The tritium overflow per 5 min fraction (●—●) and the estimated spontaneous overflow (O O) are presented. The stimulation-induced tritium overflow was obtained by subtracting the estimated spontaneous overflow from the total tritium overflow between 30 and 40 min. The stimulation-induced overflow was also expressed as per cent of the total tritium content of the tissue at the onset of stimulation.

Results

Cerebral cortex slices were incubated with ³H NA or ³H 5-HT and neostriatal slices incubated with ³H DA (10^{-7} M) for 30 min. The slices were then superfused with buffer (containing the drug to be tested) for 30 min and stimulated by an electrical field for 2 min. The tritium overflow per 5 min fraction is principally shown in Fig. 1 and in Farnebo (1971 a, b).

Cerebral cortex slices incubated with ³H NA (Fig. 2). Stimulation for 2 min greatly increased the tritium overflow from the slices (Fig. 1). The stimulation-induced overflow was 14.2 ± 0.8 %. Desipramine increased the stimulation induced overflow to about 20 %. Clonidine 10^{-7} M slightly decreased the stimulation induced overflow. Phenoxybenzamine and phentolamine 10⁻ M and 10^{-6} M increased the stimulation-induced overflow to about 25 % and chlorpromazine 10^{-6} M and 10 M to about 20 %.

Neostriatal slices incubated with ³H DA (Fig. 3). Stimulation of neostriatal slices by a current of 12 mA (as in the case of the cerebral cortex slices) caused only a slight increase of the tritium overflow. When the stimulation current was increased to 20 mA the stimulation-induced overflow was 8.7 ± 0.4 %. The stimulation-induced overflow at 20 mA was completely Ca^{++}-dependent (at zero Ca^{++} 0.3 ± 0.2 %, 4 experiments). Cocaine 10 M increased the stimulation-induced overflow to 14 %. Apomorphine caused a slight decrease of the stimulation-induced overflow. Pimozide and chlorpromazine increased the stimulation induced overflow to about 12 % while phentolamine was without effect.

Cerebral cortex slices incubated with ³H-5-HT (Fig. 4). Stimulation for 2 min increased the tritium overflow although to a lesser extent than from cerebral cortex slices incubated with ³H NA. The stimulation-induced overflow was 4.3 ± 0.3 %

During incubation of brain slices with ^{3}H monoamines only unchanged monoamines are efficiently taken up and retained in the monoamine nerve terminals. Catabolites formed during superfusion cannot accumulate in the tissue but are washed out (Rutledge and Jonason, 1967) and the spontaneous tritium overflow before stimulation is mainly catabolites (Baldessarini and Kopin 1967 Chase *et al* 1969) It is highly probable that electrical field stimulation causes release only of unchanged monoamines from the nerve terminals In brain slices incubated with ^{3}H NA or ^{3}H 5-HT the stimulation-induced tritium overflow has been shown to consist of about 70 % unchanged ^{3}H-amine (Baldessarini and Kopin 1967 Chase *et al* 1969) The increased overflow of catabolites following stimulation is likely to be due to catabolism after the release from the nerve terminals. Thus, determination of stimulation induced overflow of total tritium may be an adequate way to study release of tritiated monoamines.

Inhibition of transmitter uptake into the NA DA and 5-HT nerve terminals by desipramine, cocaine and chlorimipramine causes an increased stimulation-induced overflow In neostriatal slices cocaine may in addition to inhibition of DA uptake have a releasing effect on the DA nerve terminals (Scheel Krüger 1971) The increases of stimulation-induced overflow obtained indicate that about 35 % of the released monoamine transmitter may be taken up again into the nerve terminal The quantitative role of the reuptake mechanism for transmitter inactivation is, however difficult to establish The figure may represent the true reuptake percentage in this *in vitro* system but it may also be low due to a decreased release from the nerve terminals. It has been proposed that in the peripheral nervous system inhibition of NA uptake into the adrenergic nerves which increases receptor activation might cause a decreased NA release *via* a transsynaptic negative feed-back mechanism (Farnebo and Hamberger 1970 a, 1971 Häggendal 1970 Farnebo and Malmfors 1971)

Activation of NA DA and 5-HT receptors seems to decrease the stimulation-induced overflow Clonidine which is a potent stimulating agent of NA receptors (Hoefke and Kobinger 1966 Andén *et al* 1970 b Bolme and Fuxe 1971) slightly decreases the stimulation-induced overflow from NA nerve terminals. A decreased stimulation induced overflow of NA in the presence of clonidine has been shown in peripheral NA nerve terminals (Werner *et al.* 1970 Farnebo and Hamberger 1971) LSD-25 which is considered a potent central 5-HT receptor stimulating agent (Andén *et al.* 1968) considerably reduces the stimulation-induced overflow from 5-HT nerve terminals (Katz and Kopin, 1969) although fairly high concentrations have to be used. LSD-25 has also been shown to reduce the accumulation of 5-hydroxyindoleacetic acid upon stimulation of central 5-HT neurons *in vivo* (Randić and Padjen 1971) The DA receptor stimulating drug apomorphine (Andén *et al* 1967 b Ernst, 1967) tends to decrease the stimulation-induced overflow from DA nerve terminals Thus activation of NA, DA and 5-HT receptors seems to decrease transmitter release from the respective monoamine nerve terminals, possible *via* a transsynaptic negative feed back mechanism, see above.

Blockade of CA receptors increases the stimulation-induced overflow from NA and DA nerve terminals. Phenoxybenzamine phentolamine and chlorpromazine blocks central NA receptors (Andén *et al.* 1967, Dairman *et al.* 1968, Fuxe personal communication). All these drugs increase the stimulation-induced overflow from NA nerve terminals. Phentolamine does not inhibit NA uptake and the increase after phentolamine is thus probably due to an increased release of NA from the nerve terminals, which may be accomplished *via* a transsynaptic feed-back mechanism (*cf.* Häggendal 1970, Farnebo and Hamberger 1971). Phenoxybenzamine and chlorpromazine in addition to blockade of central NA receptors, also cause inhibition of neuronal uptake of NA although less potently than desipramine (Dengler *et al.*, 1961, Iversen 1965). The increased stimulation-induced overflow with phenoxybenzamine and chlorpromazine may thus be due to both inhibited reuptake of NA and increased release of NA from the nerve terminals. Pimozide and chlorpromazine are potent blocking agents of central DA receptors (Carlsson and Lindqvist 1963, Janssen *et al.* 1968, Andén *et al.*, 1970 a) and increase the stimulation-induced overflow from DA nerve terminals without greatly inhibiting DA uptake (Häggendal and Hamberger 1967, Hamberger 1967). These results may be interpreted as being due to an increased DA release from the DA nerve terminals. However results obtained from field stimulation of neostriatal slices must be interpreted with great caution due to the pronounced changes in the fluorescence histochemical appearance of the neostriatal slices during incubation at 37° C (Farnebo 1971 a).

The effect of drugs on release from NA, DA and 5-HT nerve terminals show a common pattern where receptor activation leads to a decreased transmitter release while receptor blockade leads to an increased transmitter relase. A similar pattern has been found in the release of NA from peripheral adrenergic nerve terminals. Furthermore phentolamine does not influence the stimulation-induced overflow from DA nerve terminals and neither chlorpromazine nor phentolamine influence the overflow from 5-HT nerve terminals. Drugs tested in the present investigation influence the rate of synthesis and turnover of monoamines in the central nervous system. Receptor activating drugs are known to decrease turnover and receptor blocking drugs are known to increase turnover (for references see Introduction). These effects are nerve impulse dependent (Andén *et al.* 1967 a, 1971, Nybäck and Sedvall, 1971) and are generally considered to be secondary to changes in nerve impulse frequency in the monoamine neurons *via* a nervous feed-back mechanism (Carlsson and Lindqvist, 1963). The increased fluorescence intensity found in central CA nerve cell bodies after CA receptor blocking drugs has also been taken as support for this view (Andén *et al.* 1966). However the probability that intact nervous loops exist in brain slices used in this study seems to be very small. Thus it seems probable that the turnover changes found *in vivo* after these drugs may at least partly be due to a *local* effect at the central monoamine synapse possibly via a negative feed-back mechanism.

Acknowledgements

This investig tion has been supported by research grants from the Swedish Medical Research Council (B7 14X-2330-05A) and Magnus Bergvalls Stiftelse For skilful technical assistance we are much indebted to Miss Ulla Enberg and Mrs Annika Hamberger

References

ANDÉN N E., A. CARLSSON A DAHLSTRÖM K. FUXE, N Å. HILLARP and K. LARSSON Demonstration and mapping out of igro-neostriatal dopamine neurons. *Life S* 1964 a. *3* 523—530

ANDÉN N E., B.-E. ROOS and B. WERDINIUS, Effects of chlorpromazine haloperidol and reserpine on the levels of phenolic acids in rabbit corpus striatum. *Lif Sci* 1964 b. *3* 149—158

ANDÉN N E A DAHLSTRÖM K. FUXE and T HÖK ELT Th effect of haloperidol and chlor promazine on the amine levels of central monoamine neurons. *Act phy iol sc d* 1966. *68* 419—420.

ANDÉN N E H. CORRODI, K. FUXE and T HÖKFELT Increased impulse flow in bulbospinal noradrenaline neurons produced by catecholamine receptor blocking agents. *Europ J Pharm ol.* 1967 59—64

ANDÉN N E., A RUBENSON K. FUXE and T HÖKFELT Evidence for dopamine receptor stimulation by apomorphine *J Ph rm Pharmacol.* 1967 b. *19* 627—629

ANDÉN N E., H CORRODI K. FUXE and T HÖK ELT Evidence for a central 5-hydroxy tryptamine receptor timulation by lysergic acid diethylamid *Brit J Pharmacol* 1968 *34* 1—7

ANDÉN, N E., S. G BUTCHER, H CORRODI, K. FUXE and U UNGERSTEDT Receptor activity and t rnover of dopamine nd noradrenaline fter neuroleptics. *E p J Pharmacol* 1970 a. *11* 303—314

ANDÉN, N E., H CORRODI K. FUXE, B. HÖKFELT T HÖK ELT C. RYDIN nd T SVENSSON Evidence for central noradrenaline receptor timulation by clonidine *Lif Sci.* 1970 b. *9* 513—523

ANDÉN N E H. CORRODI K FUXE and U UNGERSTEDT Importance of nervous impulse flow for the neuroleptic induced increase in amine turnover in central dopamine neurons. *Eu op J Pharm col* 1971 *15* 193—199

BALDESSARINI, R. J and I J KOPIN The effect of drugs on the release of norepinephrine H³ from central nervous system tissues by electrical stimulation vitro *J Pharmacol p Ther* 1967 *156* 31—38.

BESSON M J A. CHER MY and J GLOWINSKI Effects of some psychotropic drugs on dopamine synthesis in the rat striatum *J Pharmacol. xp Ther* 1971 *177* 196—205

CARLSSO A. and M. LINDQVIST Effect of chlorpromazine or haloperidol on formation of 3-methoxytyramine and normetanephrine in mouse brain *Act Pharm col. T xicol.* 1963 *20* 140—144

CARLSSON A K. FUXE, B. H MBERGER and M. LINDQVIST Biochemical and histochemical tudies on the effects of imipramine-like drugs nd (+)-amphetamine on central and peripheral catecholamine neurons. *Acta physiol. scand* 1966. *67* 481—497

CARLSSON A Structural specificity f inhibition of C-5-hydroxytryptamine uptake by cerebral slices. *J Pharm. Pharmacol.* 1970 22 729—732.

CHASE, T N R. I K TZ and I J KOPIN, Release of ³H-serotonin from brain slices. *J Neurochem* 1969 *16* 607—615

CORRODI, H and K. FUXE Decreased turnover in central 5-HT nerve terminals induced by antidepressant drugs of th imipramine type. *Europ J Pharmacol.* 1969 7 56—59

CORRODI, H., K. FUXE and T HÖKFELT The effect of neuroleptics on the activity of central catecholamine neurones. *Lif Sci.* 1967 a. 6 767—774

CORRODI, H K. FUXE and T HÖKFEL The effect of some psychoactive drugs on central monoamine neurons. *Eu p J Pharmacol.* 1967 b. *1* 363—368

DAIRMAN W R. GORDON S SPECTOR, A SJOERDSMA and S. UDENFRIEND Increased synthesis of catecholamines in th intact rat following administration of α-adrenergic blocking agents. *Mole Pharmacol.* 1968. *4* 457—464

DENGLER, H. J H. E SPIEGEL and E. TITUS, Effects of drugs on uptake of isotopic nor epinephrine by cat tissues. *Natu* 1961 *191* 816—817

DENGLER, H J I A. MICHAELSON H E. SPIEGEL and E TITUS, The uptake of labelled nor epinephrine by isolated brain and other tissues of the cat. *Int J Neuropharmacol.* 1962. *1* 23—38

ERNST A. M Mode of action of pomorphine and dexamphetamine on gnawing ompul ion in rats. *Psychopharmacol gia* (Berl.) 1967 *10* 316—323
FARNEBO, L.-O Histochemical demonstration of transmitter release from noradrenaline dopamine and 5-hydroxytryptamine nerv terminals in field stimulated at br in lices. *Z Z Hforsch* 1971 a *172* 503—519
FARNEBO L.-O R lease of monoamines evoked by field timulation. — Studies n some ionic and metabolic requirements. *Act physiol. scand* 1971 b Suppl 371 19—27
FARNEBO L.-O and B HAMBERGER Effect of desi amine phentolamine d phenoxy benzamine on the release of noradrenaline from isolated tissues. *J Pharm Ph macol* 1970 a. 22 855—857
FARNEBO, L.-O and B. HAMBERGER, Release of norepinephrine from isolated rat iris by field stimulation. *J Pharm ol. p Th* 1970 b. *172* 33 —311
FARNEBO, L.-O and B. HAMBERGER Drug induced hanges in the release of H noradrenaline from field stimulated r t iris *Brit J Ph macol* 1971 *43* 97—106
FARNEBO L.-O and T MALMFORS H Norad enaline release and mech nical response in the field stimulated mouse as deferens. *Acta phy iol. sc d* 1971 Suppl. 371 1—18.
FUXE, K., Evidence f the existenc of monoamine neurons the entral ervous system. IV Distribution of monoamine nerv terminals in the centr l nervous system. *Act physiol. scand* 1965. *64* Suppl. 17 39—85
FUXE, K., B. HAMBERGER and T HÖKFELT Distribution of noradrenal'ne nerve terminals i cortical areas of the rat. *B ai R* 1968 *8* 125—131
GEY K. F and A. PLETSCHER, Acceleration of t rnover of ¹⁴C-catecholamines in rat brain by chlorpromazine *Experientia* (Basel) 1968. *4* 335—336
HÄGGENDAL J Some f rther aspects on the release of the adrenergic transmitter I *New A pe ts of Storag and R l ase M hanisms of C t holamines*. Bayer Symposium II (Ed.: H. J Schümann and G Kroneberg) Springer Verlag Berlin—Heid lberg—New York. 1970. pp. 100—109
HÄGGENDAL, J and B. HAMBERGER, Quantitative *vitro* studies on noradrenaline uptake and its inhibition by amphetamine desipramine and chlorpromazine *Acta physiol. scand* 1967 *70* 277—280.
HAMBERGER, B., Reserpine-resistant ptake of c techolamines in isolated tissues of the rat. A histochemical study *A t physiol. sc nd* 1967 Suppl 295 1—56.
HEDQVIST P Studies on the effect of prostaglandins E and E₂ on the sympathetic euro-muscular transmission i some nimal tissues. *Act phy iol. scand* 1970 Suppl. 345 1—40
HOEFKE, W and W KOBINGER, Pharmakologische Wirkungen des 2 (2,6-Dichlorophenyl-amino) 2-imidazolin hydrochlorid, einer neuen antihypertensiven S bstanz. *Arzneimittel-Forsch.* 1966. *16* 1038—1050
IVERSEN, L. L. The inh bition of noradrenaline uptak by drugs. *Ad Drug Res* 1965 2 5—23
JANSSEN, P A. J C. J E. NIEMEGEERS, K. H L. SCHELLEKENS, A. DRESSE, F M. LENAERTS, A. PINCHARD, W K. A. SCHA ER, J M. VAN NUETE and F J VERBRUGGEN, Pimozide, chemically nov l, highly potent and orally long-acting neuroleptic drug. *Arzneim ttel-Forsch* 1968. *18* 261—287
JONSSON G K. F XE, B. HAMBERGER nd T HÖKFE 6-Hydroxytryptamine — new tool in monoamine fluorescence histochemistry *Brain R s*. 1969 *13* 190—195
KAO C. Y Tetrodotoxin, saxitoxin and their significance i th tudy of excitation phenomena. *Pharmacol. Rev* 1966. *18* 997—1049
KATZ, R. L nd I. J KOPIN Effect of D-LSD and related compounds on release of nor epinephrine H and serotonin-H³ evoked from brain slices by electrical stimulation. *Pharma ol. Res Comm* 1969 *1* 54—62.
LIDBRINK, P G JONSSON nd K. FUXE, The effect of imipramine-like drugs and antihistamine drugs on uptak mechanisms in the central noradrenaline and 5-hydroxytryptamine neurons. *Neu pharma ol* 1971 *10* 521—536.
McILWAIN, H and R RODNIGHT *P actical Neurock mistry* J A. Churchill, London, 1962.
MEEK, J and B WERDINIUS Hydroxytryptamine turnover decreased by the antidepressant drug chlorimipramine *J Pharm Pharmacol* 1970 *22* 141—143
NYBÄCK, H Z BORZECKI and G SEDVALL, Accumulation and disappearance of catecholamines formed from tyrosine-¹⁴C in mouse brain Effects of some psychotropic drugs. *E p J Pharmacol.* 1968. *4* 395—403
NYBÄCK, H J SCH BERT and G SEDVALL, Effect of apomorphine and pimozide on synthesis and turnover of labelled catecholamines in mouse brain. *J Pharm. Pharmacol.* 1970 *22* 622—624
NYBÄCK, H. and G SEDVALL, Effect of nigral lesion on chlorpromazine-induced acceleration of dopamine synthesis from ¹⁴C-tyrosine. *J Pharm. Pharmacol.* 1971 *23* 322—326

RANDIC, M. and A. PADJEN Effect of N N-dimethyltryptamine and D-lysergic acid diethyl-amide on the release of 5-hydroxyindoles in rat forebrain. *Nature* 1971 *230* 532—533
ROSS S B and A. L. RENYI, Accumulation of tritiated 5-hydroxytryptamine i brain slices. *Life Sci* 1967 *6* 1407—1415
RUTLEDGE, C. O and J JONASON Metabolic pathways of dopamine and norepinephrine in rabbit brain *in vitro* *J Pharmacol Exp Ther* 1967 *157* 493—502.
SCHEEL KRÜGER, J Behavioural and biochemical comparison of amphetamine derivatives cocaine benztropine and tricyclic antidepressant drugs. *Europ J Pharmacol* 1971 (in press)
SCHUBERT J H NYBÄCK and G SEDVALL, Accumulation and disappearance of ³H 5-hydroxy-tryptamine formed from ³H tryptophan in mouse brain Effect of LSD-25 *Europ J Pharmacol.* 1970. *10* 15—224
SHASKAN, E. G and S. H SNYDER, Kinetics of serotonin accumulation into slices from rat brain relationship to catecholamine uptake. *J Pharmacol. Exp Ther* 1970 *175* 404—418
WERNER, U., K. STARKE and H J SCHÜMANN, Effect of clonidine (ST 155) and Bay a6781 on the isolated rabbit heart. *Naunyn-Schmiedeberg Arch. exp Path Pharmak* 1970 *266* 474—475

Acta physiol. scand. 1971 Suppl 371 45—52

From the Department of Histology Karolinska Institutet, S-104 01 Stockholm 60 Sweden

Effect of d Amphetamine on Spontaneous and Stimulation Induced Release of Catecholamines

By

Lars-Ove Farnebo

Abstract

Farnebo L.-O *Effect of d-amphetamine on spontaneous and stimulation-induced release of catecholamines* Acta physiol. scand 1971 Suppl 371 45—52

The effect of d-amphetamine on the uptake spontaneous release and stimulation induced release of catecholamines (CA) in central and peripheral tissues was investigated *in vitro* d-Amphetamine was most potent to cause release of extragranularly located CA, taken up after pretreatment with reserpine and nialamide In agreement with previous investigations it is concluded that this effect is probably mainly responsible for the well-known central stimulating action of the drug at low doses. About ten times higher concentration was needed to reduce uptake and retention of CA in tissues of untreated rats. At about the same concentration increase of the stimulation-induced tritium overflow was obtained both from NA and DA nerve terminals. This increase might contribute to the CA depletion caused by high doses of d-amphetamine *in vivo* Spontaneous CA release from tissues of untreated rats was affected only at high concentration.

Introduction

The central stimulating action of d-amphetamine in mammals is indirect mediated via brain CA[1] (Stein, 1964) The behavioural effects of d amphetamine are abolished if synthesis of CA is blocked by inhibition of tyrosine hydroxylase (Weissman and Koe 1965 Randrup and Munkvad 1966 Weissman *et al.*, 1966 Hanson 1967) The stimulating effect is restored upon administration of L-DOPA (Randrup and Munkvad, 1966 Hanson, 1967)

Several effects of d-amphetamine have been demonstrated on central CA nerves. The mechanism which is mainly responsible for the behavioural effects of the drug is not fully established (see Iversen, 1967) d-Amphetamine prevents accumulation of exogenous CA in NA and DA nerves (Axelrod *et al.*, 1962 Carlsson *et al.*, 1966 Glowinski and Axelrod 1966 Hamberger 1967) and causes release of CA from the nerves (Axelrod *et al.*, 1962 Carlsson *et al.*, 1966 Glowinski and Axelrod 1966). CA located extragranularly in the axoplasm are more easily released by d-amphet-

[1] Abbreviations used CA = catecholamine( ); DA = dopamine; L-DOPA = L-dihydroxyphenylalanine NA = noradrenaline MAO = monoamine oxidase.

mine than CA in the amine storage granules (Carlsson *et al* 196.. 1966 Lundborg 1969 Fuxe and Ungerstedt, 1970) d Amphetamine also seems to increase nerve stimulation-induced CA release both in the peripheral (Obianwu 1969 Loffelholz and Muscholl, 1970) and central nervous system (Ungerstedt, 1971) In addition, d-amphetamine inhibits deamination of CA (Blaschko *et al* 1937) but this effect seems to be due to reduced intraneuronal accumulation of CA (Rutledge 1970) The aim of the present investigation was to study the effect of d-amphetamine *in vitro* on the uptake spontaneous release and stimulation-induced release of CA in central and peripheral tissues, in order to evaluate the relative importance of the different actions of d-amphetamine at various concentrations.

Material and Methods

Adult female albino rats (Sprague-Dawley 180—200 g) were used. Some of the animals were pretreated with reserpine (10 mg/kg *i.p.*) 16 h before death and/or nialamid (100 mg/kg *i.p.*) 1—2 h before death The animals were bled out under ether anesthesia and the eyes and the brain rapidly dissected out. Isolated irides (including the ciliary bodies) were prepared (Malmfors, 1965) and immediately immersed in cool Krebs-Ringer bicarbonat buffer The brain was chilled in buffer for a few min, and round tissue slices (diameter 3 mm, weight about 5 mg) were then prepared from the parietal cerebral cortex and the neostriatum using a razor blad a frosted object slide and cylindrical punch (McIlwain and Rodnight, 196 Hamberger 1967)

Upt k f CA Isolated irides or neostriatal slices of untreated animals were preincubated for 15 min t 37 C in drug-free buffer or buffer containing d-amphetamine 10 M – 10^{-4} M. ^{3}H-CA was then added to the incubation flasks t give final concentration of 10^{-7} M ^{3}H-NA (tw irides in 1 ml) or ×10 M ^{3}H DA (two neostriatal slices in 5 ml) and the incubation was continued for 30 min. The tissue was then rinsed in 5 ml fresh buffer for 10 ml and subsequently solubilized in 0.5 ml Soluene® Total radioactivity was determined in a Packard 3320 Liquid Scintillation Spectrometer after the addition of 10 ml toluene scintillation solution

Spo ta eous lease f CA Isolated irides or neostriatal slices from untreated rats or rats pretreated with reserpine and nialamid were incubated for 30 min t 37 C with ^{3}H-NA 10 M or ^{3}H DA 2×10 M, respectively Th tissue was after rapid rinsing in fresh buffer reincubated for 30 min at 37 C in 5 ml drug free buffer or buff containing d-amphetamine 10 M – 10^{-4} M. The tissue was then solubilized and radioactivity determined as above

Stimulation-i d d leas of CA Isolated irides, cerebral cortex slices or neostriatal slices from untreated rats were incubated for 30 min at 37 C with ^{3}H NA 10 M (isolated irides, cerebral cortex slices) or ^{3}H DA 10^{-7} M (neostriatal lices) The tissue was then transferred to small chambers where it was superfused by drug-free buffer (0.5 ml/min) for 30 min. d-Amphetamine 10 M – 10 M was then added to the buffer and superfusion continued for 20 min, before the tissue was stimulated by an electrical field generated between platinum lectrodes at the two ends of the chamber [biphasic pulses, 10 Hz, 2 msec, 12 mA (isolated irides, cerebral cortex slices) or 20 mA (neostriatal slices) see Baldessarini and Kopin, 1967 Farnebo and Hamberger 1970 1971 a, b]. The irides were stimulated for 10 min and further superfused for 15 min, while the brain slices were stimulated for 2 min and further superfused for 15 min. The superfusate was collected in 5 min fractions directly in vials for liquid scintillation counting and total radioactivity determined after addition of 5 ml Insta-Gel® At the end of the superfusion the tissue was solubilized and radioactivity determined as above.

Stimulation induced overflow (in dpm) was calculated by subtracting th estimated spontaneous tritium overflow from the total tritium overflow during timulation. Stimulation-induced overflow was also expressed as per cent of the total tissu triti m at the onset of stimulation (see Farnebo and Hamberger 1971)

Substances used dl-noradrenaline 7 H HCl (5— 10 Ci/mmole Radiochemical Centre, Amersham) dopamine-7 ^{3}H HB (5— 10 Ci/mmole New England Nuclear Corp., Boston) reser pine (Serpasil® Swedish Ciba) nialamide (Niamid® Swedish Pfizer) d-amphetamin sulphate (Sigma) Soluene® and Insta-Gel® (Packard)

TABLE I Effect of d-amphetamine on CA uptak and etention *in tro*

Concentration of d-amphet mine	Irides	Neostriatal slices
Control	100 ± 4 (8)	100 ± 6 (8)
10[illegible] M	100 ± 9 (4)	113 ± 7 (8)
10[illegible] M	88 ± 8 (4)	97 ± 4 (6)
10[illegible] M	48 ± 2 (8)	51 ± 4 (4)

Isolated irides nd neostriatal slices from unt eated rats wer preincubated for 15 min t 37 C in drug-free buffer or buffer cont ining d-amphetamine 10[illegible] M—10[illegible] M H NA nd H DA wa then added t the incubation flasks containing the isolated irides (10[illegible] M) nd the neostriatal slices ($2\times10^{-}$ M) respectively nd incubation continued for 30 mi After rinsing in fresh buffer for 10 min, tot l radioactivity in the tissue was determined. The radioactivity retained per iris or slice was expressed as per ent of the drug-free control The values given re mean ± S.E.M N mber of determinations withi parentheses.

TABLE II Effect of d-amphetamine on spontaneous CA elease *in vitro*

Concentration of d-amphetamine	Irides		Neostriatal slices	
	U treated	Reserpine ialamide	U treated	Reserpine nialamide
Control	100 ± 3 (20)	100 ± 4 (15)	100 ± 3 (24)	100 ± 4 (14)
10[illegible] M	98 ± 2 (4)	64 ± 7 (8)	91 ± 8 (15)	86 ± 10 (10)
10[illegible] M	102 ± 13 (8)	38 ± 2 (11)	85 ± 7 (15)	73 ± 9 (10)
10[illegible] M	90 4 (4)	29 ± 2 (8)	69 ± 6 (11)	43 ± 5 (10)
10[illegible] M	81 ± 5 (16)		73 ± 5 (3)	

Isolated irides nd neostriatal slices from ntreated rats or rats pretreated with reserpine (10 mg/kg, *i.p.* 16 h) nd ialamid (100 mg/kg *p* 1—2 h) wer incubated for 30 min t 37° C with H NA 10[illegible] M nd H DA $2\times10^{-}$ M, respectively The tissue was fter rapid rinsing in f esh buffer reincubated for 30 mi t 37 C i drug-free buffer or buffer containing d-amphetamine 10[illegible] M—10[illegible] M, and then solubilized nd total radioactivity determined. The content of radio- ctivity per iris or slice was expressed as per cent of the drug-free control Th values given re mea ± S. E. M N mber of determina ions within paren hesis.

TABLE III Effect of d-amphet mine on stimulation-induced triti m overflow

Concentration of d-amphetamine	Per cent stimulation-induced overflow		
	Irides	Cerebral cortex slices	Neostriatal lices
Control	12.8 ± 0 6 (12)	10.5 ± 0.9 (8)	9 1 ± 1 0 (6)
10[illegible] M	14.9 ± 2.1 (10)	11.3 ± 1.0 (8)	12.5 ± 2 7 (4)
10[illegible] M	26.4 ± 2 7 (6)	15.1 ± 1.2 (8)	15.4 ± 0 6 (8)
10[illegible] M	32.5 ± 1 0 (6)	19.2 ± 1 0 (8)	12.6 ± 1 0 (8)

Isolated irides, cerebral cortex lices d neostriatal slices were prepared from untreated rats. The isola ed irides and the cerebral cortex slices were incubated with H NA 10[illegible] M and the neo- stri tal slices incubated with ^{3}H DA 10[illegible] M for 30 min t 37° C. The tissue was then superfused with drug-free buffer for 30 min, superfused with buffer with or without d-amphetamine for another 20 min and subsequently stimulated by lectrical field for 10 min (isolated irides) or 2 min (brain slices) (see Figs. 1—3) The stimulation-induced tritium overflow was expressed as per cent of the total tissue tritium at the onset of stimulation (see Material and Methods) Mea ± S E. M Number of experiments within parentheses

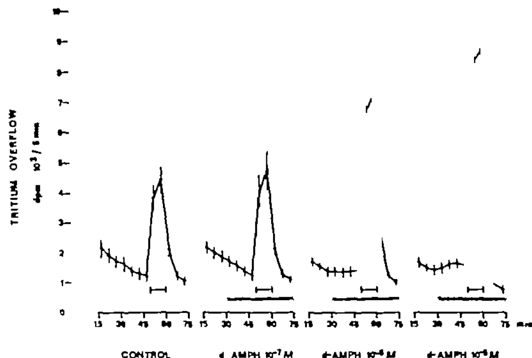


Fig 1 Effect f d-amphetamine on spontaneous and stimulation-induced tritium overflow from isolated irides of untreated rats after preincubation w th ^{3}H NA (10 M) The irides were superfused with drug free buffer for 30 min before d-amphetamine 10 M—10 M was added to the buffer ( ) After superfusion with d-amphetamine for 20 min the irides were stimulated by an electrical field for 10 min (|—|) nd further superfused for 15 min. The tritium overflow per 5 min fraction is presented. The mean tritium content at the onset of stimulation was about 50×10^3 dpm/iris. The per cent stimulation-induced overflow from these experiments is presented in Table 3

Results

Uptake of CA The retention of tritium in isolated irides and neostriatal slices after incubation with ^{3}H NA and ^{3}H DA respectively was reduced by d-amphetamine. About 50 % reducti n was obtained with d amphetamine 10^{-6} M (Table 1)

Spontaneous release of CA After incubation of isolated irides or neostriatal slices with ^{3}H NA or H DA, respectively the tissue was reincubated in buffer with or without d-amphetamine A pronounced difference in the potency of d-amphetamine to cause spontaneous release of tritium was found between tissues from untreated and reserpine nialamide pretreated rats (Table 2) In irides from rats pretreated with reserpine and nialamide d amphetamine 10^{-6} M decreased the tritium content to about 60 % of the drug free control. A similar depletion of tritium from irides of untreated rats could not be obtained even with d-amphetamine 10^{-5} M.

Stimulation-induced release of CA (Figs. 1—3) Isolated irides and cerebral cortex slices preincubated with ^{3}H NA and neostriatal slices preincubated with ^{3}H DA were superfused with drug free buffer for 30 min. d Amphetamine was then

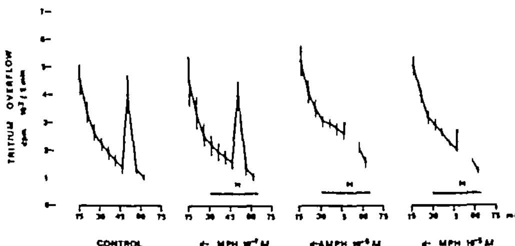


Fig. — Effect of d-amphetamine on spontaneous and stimulation-induced tritium overflow from cerebral cortex slices of untreated rats after preincubation with ^{3}H-NA (10^{-7} M). The slices were superfused with drug free buffer for 30 min before d amphetamine 10^{-7} M–10^{-5} M was added to the buffer ( ) After superfusion with d-amphetamine for 20 min the slices were stimulated by an electrical field for 2 min (|—|) and further superfused for 15 min. The tritium overflow per 5 min fraction is presented. The mean tritium content at the onset of stimulation was about 30×10^3 dpm/slice The per cent stimulation-induced overflow from these experiments is presented in Table 3

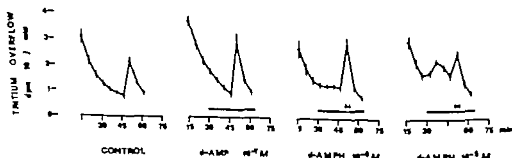


Fig. 3 Effect of d-amphetamine on spontaneous and stimulation-induced tritium overflow from neostriatal slices of untreated rats after preincubation with ^{3}H-DA (10^{-7} M). The slices were superfused with drug-free buffer for 30 min before d-amphetamine 10^{-7} M–10^{-5} M was added to the buffer ( ) After superfusion with d-amphetamine for 20 min the slices were stimulated by an electrical field for 2 min (|—|) and further superfused for 15 min. The tritium overflow per 5 min fraction is presented. The total tritium content of the slices at the onset of stimulation was about 200×10^3 dpm/slice in the drug-free control. The per cent stimulation-induced overflow from these experiments is presented in Table 3

added to the superfusing buffer This caused a slight increase of the spontaneous tritium overflow at 10^{-6} M and 10^{-5} M. Electrical field stimulation increased the tritium overflow both from the isolated irides and from the brain slices. The stimulation induced overflow was considerably increased by d-amphetamine 10^{-6} M–10^{-5} M (Table 3)

Discussion

In the present investigation a wide range of concentrations of d-amphetamine was tested in an attempt to distinguish between the various effects of the drug on CA neurons (see Introduction). No principal differences were found *in vitro* between the NA nerve terminals in isolated irides and cerebral cortex slices and the DA nerve terminals in neostriatal slices. The results are therefore discussed in general terms concerning CA.

The far most potent action of d-amphetamine via the ability to cause release of CA previously taken up in tissues of rats pretreated with reserpine and nialamide. After such pretreatment CA taken up into the nerves mainly are located extragranularly in the axoplasm (see Malmfors 1965, Carlsson, 1966, Stitzel and Lundborg 1967, Jonsson and Sachs, 1969). The present findings are entirely consistent with the observation of Carlsson *et al.* (1966) that CA, accumulated after administration of L-DOPA to animals pretreated with reserpine and nialamide are easily releaseable by low doses of d-amphetamine. The central stimulating effect of d-amphetamine is not abolished by pretreatment with reserpine (van Rossum *et al.* 1962, Svensson 1970) which depletes granular CA stores, but does not inhibit extragranular formation of DA (Andén *et al.* 1964). On the other hand the stimulating effect is abolished by tyrosine hydroxylase inhibition (Randrup and Munkvad 1966, Weissman *et al.* 1966, Hanson 1967) which seem to reduce extragranular CA stores, before granular CA stores are depleted. Thus this releasing action of d-amphetamine on extragranular CA is probably the most important one for the central stimulating action at low doses *in vivo* (Carlsson *et al.* 1966). On the other hand d-amphetamine is comparably little potent to cause spontaneous release of CA taken up into the nerves and incorporated in the amine storage granules in tissues of untreated rats and it is not likely that this mechanism plays an important role *in vivo* until very high doses of d-amphetamine are given (Fuxe and Ungerstedt 1970).

d-Amphetamine is about 10 times less potent to prevent uptake and retention of CA in tissues of untreated rats compared to its releasing effect on extragranular CA stores. The effect might predominantly be due to inhibition of CA uptake *via* the CA membrane pump but may also be due to increased spontaneous release of CA which have been taken up into the nerves but not yet incorporated into the amine storage granules. CA depletion caused by d-amphetamine *in vivo* can partially be counteracted by membrane pump inhibition (Carlsson, 1970) which seems to favour the view that d-amphetamine is at least to a certain extent accumulated in CA nerves *via* the membrane pump. However the membrane pump blocking activity of d-amphetamine is only slight at low doses, which cause clearcut behavioural effects (Carlsson *et al.* 1969).

The potentiating effect of d-amphetamine on the stimulation-induced overflow occurs at about the same concentrations that prevent uptake and retention of CA. However it does not seem likely that the increased stimulation-induced overflow

is mainly due to inhibition of reuptake of released transmitter as only a moderate increase of the stimulation-induced overflow is obtained using potent inhibitors of the membrane pump (Farnebo and Hamberger 1971 a b) In the rat iris electrical field stimulation only causes release of granularly located amines while extra granular amines are not released (Farnebo and Hamberger 1970 Farnebo 1971) d Amphetamine in higher concentrations seems to facilitate stimulation induced release of granular CA (se also Lundborg 1969) but thi might be mediated via an effect of d-amphetamine on extragranular CA The great nerve impulse-dependent increase of CA release might be responsible for the CA depletion caused by high doses of d-amphetamine *in vivo* (Moore 1963) On the other hand the nerve impulse-dependent increase of CA release seems to be of minor importance for the behavioural effects of low doses of d amphetamine (see above) which probably are dependent on release of extragranular CA

Acknowledgements

This investigation has been supported by a rese rch grant from the Swedish Medical Research Council (B7? 14X 2330-05A) Th skilf l technical assistance of Miss Ulla Enberg is gr tefully acknowledged. For generous supplies of drugs thanks re due to Swedish Pfizer (Nialamide®) and Swedish Ciba (Serpasil®)

References

Andén, N E., B. E. Roos and B. Werdinius, Effects of chlorpromazine, haloperidol and reserpine on the levels of phenolic acids in rabbit corpus striatum. *Lif Sci.* 1964 *3* 149—158
Axelrod J G Hertting and L. T Potter, Effect of drugs on the uptake and release of H³-norepinephrine in the rat heart. *N t re* (Lond.) 1962. *194* 297
Baldessarini, R. J and I J Kopin, The effect of drugs on the release of norepinephrine-H³ from central nervous system tissues by lectrical timulation *vitro. J Pharmacol. p Ther* 1967 *156* 31—38.
Blaschko, H D Richter and H Schlossman The oxidation of adrenaline and other amines *Biochem J* 1937 *31* 2187—2196
Carlsson A., Drugs which block the storage of 5-hydroxytryptamine and related amines. I *Handb d p Pharmakol.* (Ed. O Eichler and A. F rah) Springer Verlag Heidelberg—New York. 1966. *XIX* 529—59?
Carlsson A., Amphetamine and brain catecholamines. In *I ternational Symposium Amphetamines and R lated Compounds* (Ed. E. Costa and S. Garattini) Raven Press, New York 1970. 289—300
Carlsson, A M. Lindqvist A Dahlström K. Fuxe and D Masuoka, Effects of the amphetamine group on intraneuronal brain amines *in vivo* nd *in vitro. J Pharm. Pharmacol.* 1965 *17* 541—5 4
Carlsson A., K. Fuxe, B. H mberger and M Lindqvist Biochemical and histochemical studies on the effects of imipramine-like drugs and (+)-amphetamine on central and peripheral catecholamine neurons. *Acta physiol. sca d* 1966 *67* 481—497
Carlsson A., H. Corrodi, K. Fuxe and T Hökfelt Effects of some antidepressant drugs on the depletion of intraneuronal brain catecholamine stores caused by 4,α-dimethyl-metatyramine *Europ J Pharmacol.* 1969 *5* 367—373
Farnebo L.-O, Effect of reserpin on release of ³H-noradrenaline ³H-dopamine and ³H metaraminol from field stimulated rat iris. *Biochem. Pharmacol.* 1971 *20* 2715—2726
Farnebo L-O and B. Hamberger, Release of norepinephrine from isolated rat iris by field stimulation *J Pharmacol. p Ther* 1970 *172* 332—341
Farnebo, L-O and B Hamberger, Drug-induced changes in the release of H-noradrenaline from field stimulated rat iris. *Brit. J Pharmacol.* 1971 a. *43* 97—106
Farnebo, L.-O and B Hamberger, Drug-induced changes in the release of ³H monoamines from field stimulated rat brain slices. *A ta physiol. scand* 1971 b. Suppl 371 35—44

FUXE, K. and U UNGERSTEDT Histochemical, biochemical and functional studies on central monoamine neurons after acute and chronic amphetamine administration. In *International Symposium on Amphetamines and Related Compounds* (Ed: E. Costa and S. Garattini) Raven Press, New York 1970 257—288

GLOWINSKI, J and J AXELROD, Effects of drugs on the disposition of H-norepinephrine in the rat brain. Second International Catecholamine Symposium. *Pharmacol. Rev* 1966 *18* 775—786

HAMBERGER, B., Reserpine-resistant uptake of catecholamines in isolated tissues of the rat. A histochemical study *Acta physiol scand* 1967 Suppl 295 1—56

HANSON L. C. F Evidence that the central action of (+) amphetamine is mediated via catecholamines. *Psychopharmacologia* (Berl.) 1967 *10* 289—297

IVERSEN L. L. *The uptake and storage of noradrenaline in sympathetic nerves* Cambridge University Press 1967

JONSSON, G and CH SACHS, Subcellular distribution of ^{3}H-noradrenaline in adrenergic nerves of mouse atrium — effect of reserpine monoamine oxidase and tyrosine hydroxylase inhibition. *Acta physiol scand* 1969 *77* 344—357

LÖFFELHOLZ K. and E. MUSCHOLL, Der Einfluss von d-Amphetamine auf die Noradrenalinabgabe aus dem isolierten Kaninchenherzen. *Naunyn-Schmiedebergs Arch. exp Path Pharmak* 1970 *266* 393—394

LUNDBORG P Amphetamine induced release of (^{3}H)metaraminol from subcellular fractions of the mouse heart. *J Pharm Pharmacol* 1969 *21* 266—268.

MALMFORS, T Studies on adrenergic nerves. The use of rat and mouse iris for direct observation on their physiology and pharmacology at cellular and subcellular levels *Acta physiol scand* 1965 *64* Suppl. 48. 1—93

MCILWAIN H. and R. RODNIGHT *Practical Neurochemistry* J A. Churchill London. 1962.

MOORE, K. E., Toxicity and catecholamine releasing actions of *d* and *l*-amphetamine in isolated aggregated mice *J Pharmacol. exp. Ther* 1963 *142* 6—12

OBIANWU H. O Some studies on the mechanism by which d-amphetamine antagonizes guanethidine induced adrenergic neurone blockade *Acta physiol scand* 1969 *75* 102—110

RANDRUP A. and I MUNKVAD, Role of catecholamines in the amphetamine excitatory response. *Nature* (Lond.) 1966 *211* 540

ROSSUM J M. van, J B. van der SCHOOT and J A. TH HURKMANS Mechanism of action of cocaine and amphetamine in the brain. *Experientia* 1962. *18* 229—231

RUTLEDGE, C. O The mechanisms by which amphetamine inhibits oxidative deamination of norepinephrine in brain *J Pharmacol exp Ther* 1970. *171* 188—195

STEIN L., Self-stimulation of the brain and the central stimulant action of amphetamine *Fed Proc* 1964 *23* 836—850

STITZEL, R. E. and P LUNDBORG, Effect of reserpine and monoamine oxidase inhibition on the uptake and subcellular distribution of ^{3}H-noradrenaline. *Brit J Pharmacol.* 1967 *29* 99—104

SVENSSON T H The effect of inhibition of catecholamine synthesis on dexamphetamine induced central stimulation. *Europ J Pharmacol.* 1970 *12* 161—166

UNGERSTEDT U Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behaviour *Acta physiol. scand* 1971 Suppl. 367 49—68

WEISSMAN A. and B K. KOE, Behavioural effects of L-α-methyltyrosine an inhibitor of tyrosine hydroxylase. *Life Sci.* 1965 *4* 1037—1048.

WEISSMAN A B K KOE and S TENEN Antiamphetamine effects following inhibition of tyrosine hydroxylase. *J Pharmacol. exp Ther* 1966. *151* 339—352.

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 372

INSULIN SECRETION

Its Regulation by Monoamines and Acid Amyloglucosidase

By

INGMAR LUNDQUIST

LUND 1971

Kungl. Boktryckeriet P.A. Norstedt & Söner
Stockholm 1871
713220

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 372

From the Department of Pharmacology University of Lund Sweden

INSULIN SECRETION

Its Regulation by Monoamines and Acid Amyloglucosidase

By

INGMAR LUNDQUIST

Erratum

Acta physiol scand. 1971 suppl. 372
page 31 line 8 "18 min should read "5 min"
page 31 line 18 "18—19 min should read 5—6 min

Lund 1971

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 372

From the Department of Pharmacology University of Lund, Sweden

INSULIN SECRETION

Its Regulation by Monoamines and Acid Amyloglucosidase

By

INGMAR LUNDQUIST

Lund 1971

Erratum

Acta physiol. scand. 1971 suppl. 372
page 31 line 8 "18 min" should read "5 min
page 31 line 18 18—19 min" should read "5—6 min

Printed in Sweden
Studentlitteratur
Lund 1971

TO MY PARENTS

This dissertation summarizes the following papers which will be referred to by the figures 1-14 given below

1 C RERUP and I LUNDQUIST Blood glucose level in mice 1 Evaluation of a new technique of multiple serial sampling Acta endocr 1966 62 357-367

2 C RERUP and I LUNDQUIST Blood glucose level in mice 2 A quantitative study of alloxan diabetes Acta endocr 1967 54 514 526

3 C RERUP and I LUNDQUIST Non-specific reaction of current glucose oxidase preparations with glycogen and its application for glycogen determinations in tissue Acta pharmacol toxicol 1967 25, 41-53

4 I LUNDQUIST K LINDSTRAND and C RERUP Separation and characterization of a glycogenolytic enzyme from a glucose oxidase preparation obtained from Aspergillus niger Scand J clin Lab Invest, 1969 23 89-95

5 I LUNDQUIST Method for determination of acid amyloglucosidase in isolated islets of the pancreas Submitted for publication in Enzyme,

6 I LUNDQUIST and C RERUP On the development of alloxan diabetes in mice Europ J Pharmacol 1967 2, 35 41

7 I LUNDQUIST Interaction of amines and aminergic blocking agents with blood glucose regulation I β-Adrenergic blockade Europ, J Pharmacol (in press)

8 I LUNDQUIST Interaction of amines and aminergic blocking agents with blood glucose regulation II α Adrenergic blockade Europ J Pharmacol (in press)

9 R HÅKANSSON I LUNDQUIST and C RERUP On the hyperglycaemic effect of DOPA and dopamine Europ J Pharmacol 1967 1, 114-119

10 R. EKHOLM L. E ERICSON and I LUNDQUIST Monoamines in the pancreatic islets of the mouse Subcellular localization of 5-hydroxytryptamine by electron microscopic autoradiography Diabetologia, 1971 7, 339-348

11 I LUNDQUIST R EKHOLM and L E ERICSON Monoamines in the pancreatic islets of the mouse 5-Hydroxytryptamine as an intracellular modifier of insulin secretion and the hypoglycaemic action of monoamine oxidase inhibitors Diabetologia, (in press)

12 I LUNDQUIST Acid Amyloglucosidase and carbohydrate regulation I Effect of exogenous amyloglucosidase on tissue glycogen blood glucose and plasma insulin Horm Metab Res (in press)

13 I LUNDQUIST Acid amyloglucosidase and carbohydrate regulation II Acid amyloglucosidase activity in the endocrine pancreas Horm Metab Res (in press)

14 I LUNDQUIST Acid amyloglucosidase and carbohydrate regulation III The induction of sulphonylurea-stimulated insulin release and its dependence on intracellular monoamines Horm Metab Res (in press)

CONTENTS

I INTRODUCTION

The regulation of carbohydrate metabolism in mammals is dependent upon the β-cell in the islets of Langerhans because of the specific ability of this cell to synthesize and release insulin The pancreatic islets are morphologically a discrete group of cells scattered within the exocrine tissue and comprising about 1 % of the total pancreatic mass In the β-cell insulin (and/or its precursor) is stored mainly in specific cytoplasmic granules According to the emiocytosis theory (cf Lacy 1967 1970) stimulation of insulin secretion causes migration of the secretory granules towards the inner surface of the cell where the granules dissolve after fusion with the cell membrane and the solubilized hormone enters the vascular compartment However other modes of insulin secretion have been suggested e g. intracellular liberation of intragranular insulin stores by gradual dissolution of the content of the granule (Orci et al, 1969) and also a secretory mechanism which completely by-passes the granules (Like and Miki 1967 Renold 1970)

Although glucose is considered to be the main regulator of insulin secretion, a variety of other substances have been shown to stimulate or to inhibit the release of the hormone The number of such agents has become considerable during the past few years for a coverage of these general aspects on insulin secretion the reader is referred to several recent and excellent reviews (Grodsky and Forsham 1966 Frohman 1969 Mayhew Wright and Ashmore 1969 Curry 1970 Levine 1970 Renold 1970 Porte and Bagdade 1970 Lacy 1970)

The purpose of this summary is to discuss some new aspects of the insulin secreting processes in mice Results are presented from studies which show that insulin secretion is partly regulated by the influence of certain arylethylamines and by the action of acid amyloglucosidase *)

*) Terminology in the field of carbohydrases with exoamylolytic properties is confusing Exoamylolytic enzymes which attack glucose polymers predominantly linked with α-1 4-bonds such as oligoglucosides glycogen and starch, by the stepwise removal of glucose units from the non--reducing ends of the molecule are generally referred to as glucoamylase (EC 3 2 1 3) or α-glucosidase (EC 3 2 1 20) The term acid amyloglucosidase is preferred by the author to characterize both the activity of the purified fungal enzyme and the activity recorded in isolated pancreatic islets The term acid amyloglucosidase is not limited to an enzyme capable of splitting only α-1 4-bonds On the other hand, it does not cover other possible neutral glucoamylases or acid and neutral maltases not operating on glycogen at acid pH

The work was concerned with the following problems

The possible influence that extracellular monoamines*) and monoamines in the β-cell may exert on insulin secretion also the influence of catecholamines on peripheral glucose utilization

The importance of β-cell glycogenolysis in insulin secreting processes by means of hydrolytic breakdown by acid amyloglucosidase as opposed to the phosphorolytic breakdown by glycogen phosphorylase The latter reaction has previously been reported to be extremely slow (Matschinsky and Ellerman 1968)

In addition the insulin secretory capacity and the carbohydrate balance during the development and different stages of alloxan diabetes were investigated to obtain models of different diabetic conditions

II MATERIAL

Animals, Female NMRI mice (Laboratory Animal Breeding Laven Denmark and Anticimex Stockholm Sweden) or American Obese mice (AO-mice) and their lean litter mates (AN-mice) obtained from Novo Ltd Copenhagen Denmark The animals were kept on a standard pellet diet (Ferrosan AB Malmö Sweden, or Astra-Ewos Södertälje Sweden) and tap water ad libitum before and throughout all experiments Adrenalectomy was performed under ether anaesthesia by the lumbar approach. Adrenalectomized animals were kept on the standard pellet diet and given 0 9 % NaCl to drink. Hypophysectomy was performed under ether anaesthesia by the transauricular route (Falconi and Rossi 1964) Unless otherwise stated alloxan diabetes was induced in non--fasted animals by the intravenous injection of 0 44 mmoles/kg of alloxan monohydrate

Chemicals The sources of the different chemicals
Glucose oxidase 250 (high purity) "Fermcozyme" CB-B and Fermcozyme" AM (Hughes & Hughes Ltd Brentwood, England)

*) Extracellular monoamines as a term covers monoamines circulating in the blood as well as noradrenaline released locally from sympathetic nerve endings as opposed to the intra-β-cell monoamines

"Agidex" (Glaxo Laboratories Ltd Greenford England) Glucose-U^{14}C ^{14}C-5-hydroxytryptamine ^{14}C-DL-DOPA and ^{3}H-DL-5-HTP (Radiochemical Centre Amersham, England) Purified shellfish glycogen and EDTA Na_2 (British Drug Houses Ltd Poole England or Merck AG Darmstadt Germany) Maltose A R (Kebo AB Stockholm Sweden or Koch-Light Laboratories Ltd Colnbrook Bucks England) Cetylpyridinium chloride (Recip AB Stockholm Sweden) Protamine hydrochloride (Vitrum AB Stockholm Sweden) p-Chloromercuribenzoate (Sigma Chemical Corporation, St. Louis Missouri USA) Bio-Gel P 150 (Bio-Rad Laboratories Richmond Virginia, USA) Sephadex G 150 and G 25 (Pharmacia AB Uppsala Sweden) DEAE-cellulose (Serva Heidelberg Germany) Dowex 50 W-X4 (H) 200 - 400 mesh and Polyethylenglycol 20 M (Kebo AB Stockholm Sweden) o-Dianisidine base (Fluka AG Buchs Switzerland) HEPES (N 2-hydroxyethylpiperazine-N 2-ethane sulphonic acid) and turanose (Nutritional Biochemical Corporation Cleveland USA) Bovine albumin (Armour Pharmaceutical Company Ltd Eastbourne England) Collagenase (Worthington Biochemical Corporation Freehold USA) Insulin immunoassay kits (Novo Ltd Copenhagen Denmark) All other chemicals were from British Drug Houses Ltd. Poole England

Drugs The sources of the different drugs L-adrenaline and L-noradrenaline (Rhône-Poulenc Ltd Paris France or British Drug Houses Ltd Poole England) L-3 4-dihydroxyphenylalanine L-5--hydroxytryptophan, dopamine hydrochloride 5-hydroxytryptamine creatinine sulphate and 5-hydroxytryptamine hydrogen maleinate (Fluka AG Buchs Switzerland) L-isopropylnoradrenaline bitartrate and L- and D-isomers of alprenolol (Hässle AB Göteborg Sweden) Ro 4-4602 (N[1] (DL-seryl)-N²-(2 3 4-trihydroxybenzyl)hydrazine hydrochloride and alloxan monohydrate (Hoffmann-La Roche Ltd. Basle Switzerland) Nialamide (Pfizer AB Stockholm Sweden) Tolbutamide and glibenclamide (Boehringer Mannheim GmbH, Germany) NSD 1015 m-hydroxybenzylhydrazide (Ferrosan AB Malmö Sweden) Tyramine hydrochloride (Kebo AB Stockholm Sweden) Reserpine phentolamine methanesulphonate and phentolamine hydrochloride (CIBA AB Stockholm Sweden) L- and D-isomers of propranolol (ICI Ltd Macclesfield England) Glucagon free pig insulin 10 times recrystallized 25 IU/mg (Novo Ltd Copenhagen Denmark) Dexamethasone 9α fluoro-16-metylprednisolone (Fredriksberg Chemical Laboratories Ltd Copenhagen Denmark) Pargyline hydrochloride (Abbot Laboratories Ltd North Chicago Ill USA) All other drugs were from British Drug Houses Ltd Poole England

Applicability of serial blood sampling by orbital puncture for the study of blood glucose fluctuations in mice

At the investigation of factors influencing insulin secretion it was considered of importance to study the alterations in the blood glucose level With sensitive methods for blood glucose determination, repeated observations on changes in the blood glucose levels in mice would be possible over a long period The introduction of the enzymatic technique for blood glucose determinations made it possible to work with blood samples as small as 10-25 ul (paper 1) Most studies of blood glucose levels in mice had previously been performed by sampling of tail blood (Goetz et al 1954 Baird and Bornstein 1959 Brolin et al 1964 amongst others) This sampling procedure which causes considerable loss of blood was regarded not only as stressful but also as time consuming Therefore for the purpose of multiple serial blood sampling for blood glucose determinations a technique for puncture of the retrobulbar venous plexus by commercially available constriction pipettes was evaluated (paper 1) The orbital bleeding technique was described already in 1913 by Pettit and serial blood sampling with this approach was performed by Crofford and Davis (1965) These authors claimed that serial blood sampling with this technique was not accurate because of a stress which influenced the blood glucose level However it was found (paper 1) that after practice and strictly controlled animal handling this technique gave very reproducible results with a precision permitting the detection of blood glucose level changes of about 15 mg/100 ml or more with very high statistical significance in a group of 5 normal mice The variations observed in the blood glucose levels in adrenalectomized and alloxan diabetic animals were as could be expected larger than in normal mice (papers 1 and 2) The handling of the animals the blood sampling and the injection resulted in changes in blood glucose levels in some of the experiments These changes although slight were sometimes significant owing to the precision of the method Therefore in all studies on blood glucose changes control groups were used and the changes were expressed as the difference between experimental and control values

Method for tissue glycogen determination

Tissue glycogen changes occur often as a consequence of fluctuations in the plasma insulin level Of the numerous methods for glycogen

determination reported in the literature acid hydrolysis of glycogen followed by the determination of glucose (Good Kramer and Somogyi 1933 Fong Schaffer and Kirk 1953 amongst others) or the direct reaction of glycogen with the anthrone reagent (Seifter et al. 1950 Carroll Longeley and Roe 1956 amongst others) have been most frequently used Acid hydrolysis of glycogen has been combined with different chemical procedures for the determination of the glucose formed Of the different methods for determining glucose preference was given to the one using the enzyme glucose oxidase (paper 1) because of its ease of performance sensitivity and claimed high specificity (Huggett and Nixon 1957 Middleton and Griffiths 1957 Marks 1959) During the evaluation of a method for the measurement of glycogen by means of acid hydrolysis followed by the glucose oxidase procedure it was observed that the glucose oxidase reagent reacted quantitatively with glycogen even without hydrolysis The most likely explanation of this phenomenon was that glycogen was converted directly into glucose by enzyme(s) present in the commercial glucose oxidase reagent This finding led to the development of a new rapid and sensitive method for glycogen determination (paper 3)

In short, The tissue specimen is digested in 30 % KOH in a boiling water bath the glycogen is fractionated with ethanol (1 5 ml pure ethanol/ml KOH) and the tube is then centrifuged decanted and allowed to drain The precipitated glycogen is dissolved in a suitable volume of water depending on the expected glycogen level e g for liver tissue 1 ml water/10 mg of wet liver and a small sample 25 – 100 μl is withdrawn and added to 3 ml of glucose oxidase reagent (Marks 1959) The reagent contains crude glucose oxidase peroxidase and o-tolidine in an acetate buffer pH 5 0 Purified shellfish glycogen is used as reference standard Colour development is allowed to proceed for 10 min and the sample is read at 625 nm Because acid hydrolysis of glycogen did not give total recovery (paper 3) and because the anthrone method gives occasional variations in colour yield of replicate samples (unpublished) the above--summarized method is thought to be more accurate than other available methods It is worth noting that methods for glycogen measurement based on treatment of a tissue homogenate with commercially available preparations of amylolytic enzymes (cf Huijing 1970) do not measure glycogen alone but also oligoglucosides of different chain length.

It was shown later (paper 4) that the crude glucose oxidase preparation ("Fermcozyme" CB B) contained an amyloglucosidase of high activity Therefore this glucose oxidase preparation is recommended for the purpose of glycogen determination (paper 3) Most commercial glucose oxidase preparations currently available are less useful since they are more pure and thus contain very small amounts of amyloglucosidase

Purification and characterization of fungal acid amyloglucosidase

The presence in crude glucose oxidase preparations of an enzyme (or enzymes) with the ability to split glucose from glycogen is of interest not only for glycogen assays (paper 3) but also because this amylolytic type of enzymes present in crude fungal extracts has been shown to penetrate liver cells when given to animals (Cuthbertson Fleming and Rice 1967) and man (Hug and Schubert 1967) Therefore it was decided to purify the enzyme(s) from the crude glucose oxidase preparation Fermcozyme" CB-B obtained from Aspergillus niger (paper 4) and later from a crude preparation of amyloglucosidase "Agidex" (paper 12) also obtained from Aspergillus niger For separating glucose oxidase which has a molecular weight of about 150 000 (Keilin and Hartree 1948) from amyloglucosidase which has a molecular weight of about 100 000 (Pazur and Kleppe 1962) gel filtration on Sephadex G 150 was found to be suitable especially when the G 150 column was provided with a top layer of G 25 (paper 4) No separation was achieved on Bio-Gel P 150 This discrepancy is interesting as Sephadex being a glucose polymer apparently retained the amyloglucosidase through the formation of an enzyme-substrate complex A similar retention on dextran gels has also been reported for other amylolytic enzymes such as pancreatic amylase (Gelotte 1964) and liver α-glucosidase (Auricchio and Sica 1967) The purified amyloglucosidase was found to have an acid pH-optimum (pH 4 6) with both glycogen and maltose as substrate at a concentration of 0 18 % (paper 4) Heat inactivation experiments revealed that the glycogen and maltose hydrolyzing activities decreased in parallel suggesting the presence of a single enzyme Furthermore thin-layer chromatography of glycogen digests showed that glucose was the only low molecular reaction product which suggested absence of endoamylolytic contaminants Kinetic studies with the purified enzyme using maltose as substrate allowed calculation of a Km = 1 22 mM at pH 4 6 With glycogen the rate of hydrolysis did not follow ordinary Michaelis-Menten kinetics The rate of glycogen hydrolysis could be roughly estimated to about 10 times that of maltose hydrolysis Furthermore it was found that the enzyme was capable of splitting not only α-1 4-bonds but also α-1 6--bonds since 95 % of glycogen was degraded upon prolonged incubation The purified enzyme could be characterized as an acid amyloglucosidase having properties similar to a previously described preparation of amyloglucosidase from Aspergillus niger (Pazur and Ando 1959 1960) These authors showed that the initial attack of the amyloglucosidase results in the stepwise liberation of glucose units from the nonreducing end of a polyglucoside molecule

The intention was to use the purified enzyme for pharmacological purposes i e to study its effects after administration to animals (mice) However the amount of enzyme obtained by purification of glucose oxidase preparations was found to be insufficient for this purpose Therefore a purification procedure was devised with "Agidex" as the source of the acid amyloglucosidase (paper 12) The crude enzyme preparation was adsorbed on a DEAE-cellulose column at pH 6 0 and eluted by a discontinuous gradient of increased salt concentration and lowered pH. The constituents of the crude enzyme preparation eluted in the last step (0 05 M citrate-phosphate pH 2 8 containing 0 5 M KCl) were concentrated and further eluted on a Sephadex G 150 column In this way a highly purified acid amyloglucosidase was obtained The enzyme was electrophoretically pure (polyacrylamide gel veronal buffer pH 8 6 stained with Coomassie blue) and the only low molecular reaction product found after glycogen digestion was glucose as judged by thin-layer chromatography on Kieselguhr G plates butanol/2 6-lutidine/water 6 3.1 v/v/v

Method for the determination of acid amyloglucosidase activity in isolated pancreatic islets

During the evaluation of the effects exerted by administered acid amyloglucosidase in mice (paper 12) it became of interest to investigate whether there was any endogenous acid amyloglucosidase activity in the pancreatic islets Therefore a method was developed for the determination of acid amyloglucosidase activity in isolated mouse islets (paper 5) The procedure used for the preparation of isolated islets was based on that of Moskalewski (1965) Pancreatic slices were incubated with collagenase in a modified Hanks solution supplied with fructose instead of the recommended glucose A low concentration of collagenase was used to minimize cell damage The islets were harvested one by one by means of a cataract knife and a pair of watch-makers forceps which was used only to "push the collagenase-liberated islets onto the cataract knife Small petri dishes on black paper containing Hanks solution at +4° C and a stereo-microscope were used Before examination, the islets were washed repeatedly in the modified Hanks solution to remove all collagenase The islets were transferred to another petri dish for a further washing and then homogenized in a Potter-Elvehjem tube containing 300-600 µl of an acetate-EDTA buffer (1 1 mM EDTA, 5 mM acetate pH 5 0) Assay of acid amyloglucosidase activity was then performed in small conical test tubes containing 25 µl homogenate 25 µl glycogen-buffer solution, and 5 µl Triton X-100 The assay conditions were as follows temperature 37° glycogen concentration 1 % (w/v) 0 05 M

sodium acetate buffer 0 5 mM EDTA 0 5 % Triton X-100 0 25 M mannitol pH 5 0

Glycogen was preferred to maltose as substrate in the assay for several reasons Glycogen is by definition a specific substrate for an amyloglucosidase though up to now all mammalian amyloglucosidases described have been shown also to split maltose (cf Smith Taylor and Whelan 1968) However it was observed (paper 5) that islet glycogen hydrolyzing activity and islet maltose hydrolyzing activity had different pH-patterns after heat inactivation which might suggest the presence of two different enzymes preferentially attacking either glycogen or maltose Thus changes in glycogen hydrolyzing activity do not necessarily involve similar changes in maltose hydrolyzing activity Another difference between the two substrates was that maltose in contrast to glycogen caused substrate inhibition of enzyme activity at concentrations of 0 5 % and higher (paper 5) Preliminary experiments had revealed endoamylolytic activity in the islets (unpublished) therefore EDTA was included in the assay to prevent the influence of endoamylolytic activity All endoamylolytic enzymes are believed to be calcium metallo-enzymes (cf Greenwood and Milne 1968) therefore this precaution was considered appropriate although no influence of EDTA was normally seen at pH 5 0 A marked inhibitory effect of EDTA on endoamylolytic activity however was recorded at pH 5 5 - 6 3 (paper 5) Triton X-100 markedly enhanced the activity of the enzyme because this detergent makes available the full activity of this particle-bound enzyme (paper 13) Mannitol was included in the assay procedure because it improved the homogenate dilution curve which for unknown reasons was recti-linear only within a rather narrow range (paper 5) One unit of acid amyloglucosidase activity is defined as the activity that liberates 1 μmole glucose/min from glycogen under the above-mentioned assay conditions An activity of about 15 units/g of protein was found in islet tissue of the non fasted NMRI-mouse This activity was found to be twice that recorded in liver tissue (paper 13)

Blood glucose determination. Determination of blood glucose levels was routinely performed enzymatically (glucose oxidase) according to Marks (1959) with a minor modification consisting of a reduction in volume of the deproteinizing fluid (NaOH + $ZnSO_4$ in NaCl) to a total volume of 2 ml

Assay of immunoreactive insulin Radioimmunological determination of insulin in plasma or pancreatic extracts was performed according to Heding (1966) using ^{125}I-labelled pig insulin and guinea pig anti-pig--insulin

In vitro glucose utilization by isolated tissues Liver muscle and adipose tissue specimens were incubated in Krebs-Ringer bicarbonate buffer (pH 7 4) supplied with 2 mg gelatine 2 mg glucose and 0 1 μc (liver 0 3 μc) glucose U-^{14}C per ml of incubation medium The incubations were performed in a metabolic agitator (100 cycles/min) in an atmosphere of 95 % O_2 and 5 % CO_2 The amount of labelled CO_2 formed was determined according to Lyngsøe (1961) ^{14}C-lipid was isolated according to Fain Scow and Chernick (1963) ^{14}C-glycogen in adipose tissue according to Leonards and Landau (1960) and ^{14}C-glycogen in liver and muscle according to the above described procedure (KOH-ethanol fractionation) but the isolated glycogen was precipitated twice

Determination of monoamine oxidase and DOPA decarboxylase activity These activities were determined in pancreatic homogenates (0 1 M phosphate buffer pH 7 0) with the radiometric methods described by Håkanson and Owman (1965) and Håkanson (1966)

Determination of dopamine Tissue dopamine was measured fluorometrically (Carlsson and Waldeck 1958) after purification by ion exchange chromatography (Bertler Carlsson and Rosengren 1958)

Determination of protein Protein was determined according to Lowry Rosebrough and Farr (1951) Bovine serum albumin was used as standard.

Electron microscopy In the anaesthetized animal, the pancreas was fixed by perfusion with 50 ml glutaraldehyde via the ascending aorta. The perfusion solution consisted of 3 % glutaraldehyde buffered with 0 075 M sodium cacodylate pH 7 2 Pancreatic specimens were dissected out from the splenic part of the pancreas cut into small pieces and transferred to 1 % osmium tetroxide in blood-isotonic veronal acetate buffer pH 7 2 Postfixation lasted for 2 h After dehydration in ethanol the tissue was embedded in Epon and islet tissue was identified by light microscopy in 1 μ sections For autoradiography pale golden sections (about 1200 Å) were cut on an LKB Ultrotome picked up on Formvar-coated copper grids stained with uranyl acetate and lead citrate and then covered with a layer of carbon by vacuum evaporation The emulsion Ilford L4 was applied to the sections by means of a wire loop (Caro and van Tubergen 1962 Maunsbach 1966) Most grids and all grids used for quantitative analys of silver grain distribution were developed after 6 weeks in Kodak D 19 B (2 min) and fixed in Kodak F-24 fixer (2 min)

IV EXPERIMENTAL DIABETES INDUCED BY ALLOXAN

In the search for models of different diabetic conditions in mice the carbohydrate balance of animals during acute diabetogenesis and during manifest diabetes due to alloxan was studied by determination of blood glucose plasma insulin and tissue glycogen (papers 1 2 6 and 12)

A The carbohydrate balance during the acute diabetogenic phase

The diabetogenic effect of alloxan has been the subject of numerous investigations ever since the discovery that it can produce a selective necrosis of the islet β-cells (Dunn Sheehan and McLetchie 1943) (For a recent review see Rerup 1970) In normal animals a diabetogenic dose of alloxan is known to elicit an acute triphasic alteration of the blood glucose level consisting of a) an initial hyperglycaemia b) a transitory hypoglycaemia, and c) a permanent hyperglycaemia This was found to apply also in mice (papers 2 and 6) The mechanism responsible for the two first phases was unknown for a long time after its discovery The initial hyperglycaemic phase was interpreted by Dunn et al (1943) as partly a consequence of insulin deficiency but Houssay Orias and Sara (1945) explained it as a direct glycogenolytic effect on hepatic glycogen stores The present data (papers 2 and 6) show that it is due both to liver glycogenolysis and to an immediate cessation of insulin secretion The ensuing hypoglycaemic phase was interpreted by Houssay Orias and Sara (1945) to be due to low glucose production by the liver Hughes Ware and Young (1944) on the other hand suggested that this phase was due to insulin leakage from the damaged β-cells It was shown (Howell and Taylor 1967 paper 6) that this phase was the result of increased plasma insulin levels This could also be demonstrated indirectly through the injection of anti-insulin serum which abolished the hypoglycaemic phase (paper 6) The marked increase in liver glycogen levels during this phase also points to an effect of insulin (paper 6) The early permanent hyperglycaemia following the hypoglycaemic phase was characterized by the absence of or by very low levels of plasma insulin (paper 6) due to the selective destruction of the β-cells Despite a highly diabetic condition as judged from the blood glucose levels certain animals still had detectable plasma insulin levels showing that not all β-cells were destroyed The results obtained (papers 2 and 6) suggested the following models of experimental diabetes with regard to the carbohydrate balance

1) The early part of the acute hyperglycaemic phase i e 5 to 30 min

after the intravenous injection of alloxan is a model for a β-cell which is totally unresponsive to insulin releasing agents such as tolbutamide (paper 6) and corticotrophin (Lundquist and Rerup 1967) and which has a severely impaired basal insulin secretion (paper 6) It is a transitory diabetic stage with hyperglycaemia and low glycogen levels (papers 2 and 6)

3) The hypoglycaemic phase and the early phase of permanent diabetes represent a condition of profound shifts in the carbohydrate balance chiefly as a consequence of uncontrolled leakage from the damaged β-cells resulting in fluctuations in plasma insulin blood glucose and liver glycogen levels (papers 2 and 6)

B The carbohydrate balance during the chronic diabetic phase

Alloxan diabetes in mice was regarded as manifest if blood glucose levels were permanently elevated above 200 mg % In addition to the high blood glucose levels and usually non-measurable plasma insulin levels (papers 2 and 6) liver glycogen was decreased as was muscle glycogen (papers 6 and 12) Other signs of diabetes such as glycosuria polydipsia and polyuria, could be registered The severity and duration of the permanent diabetic phase in mice was found as could be expected to depend on the dose of alloxan and on the individual susceptibility to the drug (paper 2) The blood glucose level was relatively constant in individual mice during several weeks of the permanent phase but highly unstable with regard to external influences (paper 2 and unpublished)

Hypophysectomy or adrenalectomy greatly reduced the survival time of diabetic animals (paper 3) The dose of alloxan generally used (0 44 mmoles/kg intravenously) was effective in female non-fasted NMRI-mice and caused permanent diabetes in practically all these animals without any serious side-effects It is worthy of note that male animals were less susceptible to alloxan and required a larger dose (unpublished) This sex difference in alloxan sensitivity is also known from other species (Beach, Bradshaw and Blatherwick 1951) The permanent diabetic phase was stable in most mice for several weeks (paper 2) however after about 2 months a spontaneous remission of the diabetic condition could occur (Rerup 1968) The improvement of the diabetic state coincided with a restoration of the islet β-cell population (Bunnag Warner and Bunnag 1967). This was taken advantage of to obtain a colony of diabetic and subdiabetic animals with a significant number of functioning β-cells (paper 12)

The results obtained (papers 2 6 and 12) suggested the following mo[illegible]

of experimental diabetes with regard to the carbohydrate balance

1) The first 4 - 5 weeks following establishment of the permanent diabetic phase are considered as a useful model of a diabetic condition characterized by high blood glucose levels and severely impaired insulin secretion (papers 2 and 6) unresponsiveness to the hypoglycaemic action of corticotrophin (Lundquist and Rerup 1967) and low tissue glycogen levels (papers 6 and 12) although susceptible to the extrapancreatic effect of a large dose of tolbutamide (paper 6) This model may to some extent resemble juvenile human diabetes

2) The phase of spontaneous remission of the diabetic state (from about 8 weeks after alloxan injection) is characterized by a significant number of functioning β-cells (Bunnag Warner and Bunnag 1967 Rerup 1968 paper 12) and a diabetic blood glucose level (Rerup unpublished paper 12) With regard to the carbohydrate balance this model may to some extent resemble maturity-onset human diabetes

V MONOAMINES AND INSULIN SECRETION

A Extracellular monoamines adrenergic receptors and carbohydrate regulation

As long ago as 1930 Colwell and Bright suggested that infusion of adrenaline will inhibit insulin release in man The inhibitory effect of adrenaline on insulin secretion, however was not convincingly demonstrated until a few years after the introduction of the first radioimmunoassay of plasma insulin (Yalow and Berson 1960) Coore and Randle (1964) showed an inhibitory effect of adrenaline on insulin secretion by pancreatic slices in vitro and Porte et al (1966) and Kris et al (1966) reported that adrenaline infused in vivo could similarly inhibit glucose-induced insulin secretion Moreover noradrenaline infused in vivo (Porte and Williams 1966) and dopamine added in vitro (Wong et al 1967) could be shown to inhibit glucose-induced insulin release The hyperglycaemic effect of dopamine in vivo was also on indirect evidence interpreted as partly due to inhibition of insulin secretion (paper 9) Numerous studies have since convincingly demonstrated that the catecholamines are able to inhibit insulin secretion induced by a variety of stimuli (cf Porte 1969)

According to present views the basal insulin secretion is regulated

mainly by the blood glucose acting on the β-cell However other mechanisms cannot be ruled out as Porte and Bagdade 1970 and Freinkel Mager and Vinnick 1968 did not find any relation between the basal plasma insulin levels and the basal blood glucose levels Similar results were recently found in mice (unpublished) and had previously been noted with regard to serum insulin like activity (fat pad method) and blood glucose level in the rat (Lundquist 1968) In adrenalectomized rats however there was a significant correlation (Lundquist 1968) The possible implication of the adrenals in the basal regulation of insulin secretion was thus considered and as the pancreatic β-cell has been shown to have both α- and β-adrenergic receptors (cf Porte 1969) it was adopted as a working hypothesis that adrenaline has a dual action on insulin secreting mechanisms analogous to its effect on the vascular system i e not only an inhibitory action (α receptor stimulation) on insulin secretion as has been shown by several workers (cf Porte 1969 Mayhew Wright and Ashmore 1969) but also a stimulating one (β receptor stimulation) (papers 7 and 8)

L-propranolol a specific β-blocking agent decreased the basal plasma insulin levels in normal mice to about 50 % (paper 7) No effect was recorded with its structural and optical isomer D-propranolol which is practically devoid of β-blocking properties (Howe and Shanks 1966) Reduction of basal insulin secretion after treatment with racemic propranolol was observed by Gagliardino et al 1970 a and Werrbach et al, 1970 whereas no change was found by Cerasi Effendic and Luft (1969) Allison et al (1969) and Åkerblom Martin and Cingolani (1969) This discrepancy might partly be explained by the use of racemic propranolol in these studies The nutritional status of the studied individuals might also be of great significance because fasting which was used in some of the above-cited studies increases the sympatho-adrenal tone and thus in the author s opinion the threshold for α-adrenergic stimulation could already have been reached before the initiation of the experiment and the promotion of insulin secretion through β-adrenergic stimulation would be masked This is analogous to the well-known adrenergic effects on the vascular system where β-adrenergic vasodilation elicited by small concentrations of adrenaline adrenaline reversal changes into a vasoconstriction once the threshold for stimulation of the less sensitive but predominant α-adrenergic receptors has been reached As a consequence the β-adrenergic effect will be completely masked. However a dual adrenergic effect on insulin secretion does not exclude the possibility that other factors for example the basal blood glucose levels might exert a regulatory influence on basal insulin secretion Moreover β-adrenergic blockade never abolished basal insulin secretion and systemic infusion or injection of adrenaline usually revealed no

intracellular pool of glucose-6-phosphate after the β-blockade of muscle glycogenolysis that permitted an augmented glucose utilization However further experiments (paper 7) showed that incubation of muscle tissue either after L-propranolol treatment in vivo or after addition in vitro *increased $^{14}CO_2$ production from glucose-U-^{14}C compared with control* tissue This finding favoured the existence of a β-receptor in the muscle with the ability to facilitate glucose transport independent of the β-effect exerted through the glycogen phosphorylase-synthetase system (cf Sutherland and Robison 1969) Thus the hypoglycaemic effect of β-adrenergic blocking drugs such as propranolol which is seen in fasting or diabetic individuals (for references see paper 7) can be explained not only through a decreased glucooneogenesis in the liver and decreased availability of free fatty acids (Abramson and Arky 1968) but also through a marked increase of glucose utilization in muscle tissue by the combined effect of a decreased glycogenolysis and a facilitated glucose uptake

Adrenergic blockade in adipose tissue The effects of adrenergic blockade on carbohydrate regulation in adipose tissue of mice can be summarized as follows (papers 7 and 8) Adrenergic regulation of adipose tissue glycogen was found to be β-receptor mediated In vitro experiments on the adrenergic regulation of glucose utilization by adipose tissue provided data which to some extent were similar to those of adrenergic regulation of insulin secretion i e α-adrenergic stimulation inhibited and β-adrenergic stimulation promoted glucose utilization The recent suggestion by Himms-Hagen (1970) that the lipolytic response of adipose tissue to adrenergic stimulation is regulated by both α-adrenergic receptors which inhibit and β-adrenergic receptors which promote lipolysis agrees with this concept However with regard to the interaction of the mechanisms regulating glucose utilization and lipolysis in adipose tissue *it is difficult to draw any definite conclusions from the present experiments*

B Intracellular monoamines in the pancreatic β-cell

In 1963 Falck and Hellman showed that the pancreatic islets of the duck guinea pig cat dog and horse contained monoamines demonstrable by their formaldehyde-induced fluorescence (Falck et al, 1962) These amines later identified as 5-hydroxytryptamine (5-HT) and dopamine (Cegrell Falck and Hellman 1964 Cegrell 1967) where shown to be present in the pancreatic islets of many other species including man (cf Cegrell 1968) But the islet cells of the adult mouse were devoid of formaldehyde-induced fluorescence (cf Cegrell 1968) This however does not exclude the possibility that other amines not detectable with available histochemical methods or low levels of 5-HT and dopamine

normally exist in mouse pancreatic islets because the mouse islet cells were found to be equipped with the required mechanisms for uptake and decarboxylation of amino precursors and for amine storage and oxidation (cf Cegrell 1968)

A preliminary attempt was made to study the possible functional implications of these findings by investigating some effects of L-DOPA (L-3 4-dihydroxyphenylalanine) and dopamine on the blood glucose regulation in normal and alloxan diabetic mice (paper 9) It was observed that both compounds elicited a rapid rise in the blood glucose level of normal mice with a peak after 15 min The effect of dopamine vanished completely after 30 min whereas the effect of L-DOPA persisted The hyperglycaemic effect of DOPA was described already in 1927 (Hirai and Gondo 1927) With the aid of a decarboxylase inhibitor (NSD 1015) and a monoamine oxidase inhibitor (nialamide) it could be demonstrated that the hyperglycaemic effect of DOPA could be ascribed to dopamine or to a dopamine derivative because the L-DOPA-induced hyperglycaemia was greatly reduced after pretreatment with NSD 1015 whereas pretreatment with nialamide increased the hyperglycaemic response (paper 9) It was also found that the dopamine-induced hyperglycaemia was at least partly pancreatic in origin because dopamine in contrast to adrenaline (paper 7) had no effect on liver glycogen content and also failed to raise the blood glucose level in alloxan diabetic animals Moreover tolbutamide antagonized the hyperglycaemic effect of dopamine pointing to a possible interference with insulin release However with regard to the rapidity of the hyperglycaemic response elicited by dopamine there is reason to believe that this response can be partly attributed to an immediate inhibition of peripheral glucose utilization similar to that exerted by adrenaline (paper 7) This presumption is based on the observation (paper 6) that anti insulin-induced hyperglycaemia was relatively slow in onset

The uptake of dopamine by the pancreatic islets is poor compared to that of its precursor L-DOPA (cf Cegrell 1968) Thus although the hyperglycaemic effect of dopamine was considered to be partly extra-pancreatic it was still possible that the prolonged hyperglycaemia elicited by L-DOPA and the impaired dopamine-storing capacity in the pancreas of alloxan diabetic mice (paper 9) reflected a role for β-cell monoamines in the mechanism of insulin release Autoradiographic investigations with the light microscope have revealed a specific uptake of the amine precursors 5-hydroxytryptophan (5-HTP) and DOPA in the pancreatic islets of the mouse (Gershon and Ross 1960 Ritzén Hammarström and Ullberg 1965 Tjälve 1971) However studies by those authors at the light-microscope level furnished limited information about the

plasma insulin levels and a combination of L-5-HTP and a monoamine oxidase inhibitor gave contradictory results However because certain amino acids are known to possess an insulin-releasing effect it is not improbable that such an effect of e g L-5-HTP might mask the inhibitory action of the monoamine oxidase inhibitor A recent report by Frohman (1971) showing that chronic treatment of rats with monoamine oxidase inhibitors resulted in decreased plasma insulin levels supports the above findings in mice (paper 11) Frohman (1971) also reported on an acute insulin release after administration of pargyline or mebanazine as previously reported after nialamide (Gagliardino et al, 1970 b) Furthermore Frohman (1971) showed that the acute insulin release after pargyline was augmented by adrenaline These monoamine oxidase inhibitors might possibly have slight α-blocking properties which would explain their slight insulin releasing capacity especially when combined with adrenaline (see paper 8)

In conclusion the present studies (paper 11) have shown that in the β-cell the intracellular levels of monoamines such as 5-HT are able to modify insulin releasing processes in vivo Injection of the amine precursor inhibited sulphonylurea-stimulated and L-IPNA-stimulated insulin release which in the latter case could be totally suppressed by combination with a monoamine oxidase inhibitor A monoamine oxidase inhibitor alone could partially inhibit insulin release by the above compounds The inhibitory action of L-5-HTP was abolished by pretreatment with an inhibitor of aromatic amino acid decarboxylation It is suggested that the amine itself is responsible for the effects obtained

VI ACID AMYLOGLUCOSIDASE AND INSULIN SECRETION

Amylolytic enzymes producing glucose through an exoamylolytic approach are known to be present in several species of fungi of the Aspergillus (*Kerr Cleveland and Katzbeck 1951 Pool and Underkofler 1953* Barker and Fleetwood 1957 Pazur and Ando 1959 amongst others) and Rhizopus groups (Tsujisaka, Fukumoto and Yamamoto 1958 Phillips and Caldwell 1951 Pazur and Okada 1967 amongst others) Such enzymes have also been isolated from yeasts and bacteria (Hopkins and Kulka 1957 French and Knapp 1950 amongst others) and from plant sources (Takahashi and Shimomura 1968 Marshall and Taylor 1971 amongst others) The enzymes can be classified according to their preference for attacking either glycogen or maltose Amyloglucosidases (preferentially attacking

glycogen or starch) act with inversion of the configuration of the reaction product whereas α-glucosidases (preferentially attacking maltose) act with retention (cf Reese Maguire and Parrish 1968 paper 4) Furthermore the amyloglucosidases are characterized by their ability to liberate glucose by the stepwise removal of glucose units from the non-reducing ends of glucose polymers (Pazur and Ando 1959) Mammalian tissues are also known to contain significant amounts of exoamylolytic hydrolases (cf Smith, Taylor and Whelan 1968) these have been found in liver muscle kidney spleen and brain The discovery of exoamylolytic activity in islet tissue (papers 5 and 13) raised the question of whether this activity was mainly to be referred to as an amyloglucosidase or as an α-glucosidase The data of paper 5 showed that islet glycogen hydrolyzing activity and islet maltose hydrolyzing activity had different heat inactivation patterns thus suggesting that both an amyloglucosidase and an α-glucosidase might be present It was observed, however that the exoamylolytic activity found in islet tissue preferentially attacked glycogen (unpublished) showing that the amyloglucosidase activity was predominant Thus the exoamylolytic activity recorded in islet tissue with the above-discussed method (paper 5) and the activity of the highly purified fungal enzyme (papers 4 and 12) can both be characterized as representing an acid amyloglucosidase

The physiological significance of mammalian amyloglucosidases and α-glucosidases is largely unknown The discovery by Hers (1963) that an acid α-1 4-glucosidase (and an acid amyloglucosidase ?) was not present in the liver heart and skeletal muscles of children with the fatal form of glycogenosis called Pompe s disease suggested that such enzymes might play a vital role in glycogen degrading processes In spite of this discovery further investigations into the possible physiological significance of these acid hydrolases are largely lacking As far as the author is aware only reports by the Rosenfeld-group (cf Rosenfeld Popova and Orlova 1970) showing that exogenous adrenaline influenced exoamylolytic hydrolases in different tissues of the rat and a recent report by Gennser Lundquist and Nilsson (1971) who observed that the activity of acid amyloglucosidase in human foetal liver significantly increased during asphyxia, have dealt with the possible physiological implication of these enzymes

In 1951 Toreson demonstrated that human and experimental diabetes mellitus was accompanied by glycogen infiltration in the pancreatic islets Seventeen years later Matschinsky and Ellerman (1968) showed that glycogen is a normal constituent of mammalian β-cells They also reported that the glycogen phosphorylase activity in the islets of the insulin hypersecreting obese hyperglycaemic mouse (AO-mouse) was

very low These findings and the hypothesis proposed by Samols Marri and Marks (1966) that β-cell glycogenolysis may be the source of intracellular glucose or glucose metabolites acting as mediators for insulin release suggested to me that the hydrolytic as opposed to the phosphorolytic breakdown of β-cell glycogen might be of physiological significance This hypothesis is further strengthened by recent reports by Hellman Idahl and Danielsson (1969) and Hellman and Idahl (1970) showing that insulin releasing agents such as glucagon and the sulphonylurea compound glibenclamide are capable of inducing β-cell glycogenolysis

A. Effect of exogenous purified fungal acid amyloglucosidase on insulin secretion

A preliminary attempt to evaluate the possible role of hydrolytic glycogenolysis in the β-cell for insulin secreting processes was based on the observation in man (Badhuin, Hers and Loeb 1964 Hug and Schubert 1967) that fungal amylases administered as crude extracts were able to penetrate into liver cells Moreover Cuthbertson, Fleming and Rice (1967) showed that a partially purified fungal acid amyloglucosidase injected into rats was able to lower liver glycogen stores *Thus the ability of an exogenously administered macromolecule* such as acid amyloglucosidase (molecular weight about 100 000) to penetrate into cells without appreciable loss of biological activity offered unique possibilities for studying its possible physiological significance It was therefore considered worth while to administer a highly purified acid amyloglucosidase (papers 4 and 12) and to see whether it would penetrate also into the β-cell and consequently permit a preliminary answer to the question of whether hydrolytic breakdown of β-cell glycogen could be of physiological significance for insulin secreting processes The data presented in paper 12 showed that administration of a highly purified acid amyloglucosidase to normal non-fasted mice induced a slight elevation of the plasma insulin levels and a fall in blood glucose level, which invariably lasted for several hours *Moreover alloxan diabetic animals in the β-cell regenerating* phase presumed to contain raised levels of β-cell glycogen as a consequence of their hyperglycaemic condition were found to respond with markedly elevated plasma insulin levels and decreased blood glucose levels upon enzyme administration These findings together with the observation (paper 14) that islet amyloglucosidase activity in normal mice increased by about 30 % 3 h after enzyme administration suggested that the enzyme indeed penetrated into the β-cells with preserved biological activity The possibility that the rise

in amyloglucosidase activity seen in the β-cell might be due not to the presence of the enzyme within the cells but rather to the presence of the enzyme in residual blood tissue fluid or on the β-cell surface was also considered. However this is unlikely because the amyloglucosidase activity in serum had returned to control values at least 2 h before islet isolation (paper 12) Furthermore an effect on the cell membrane would not be expected to occur 3 h after enzyme injection because the half-life of the enzyme in blood is only about 18 min (paper 12) and the elevation of plasma insulin levels was not recorded until 2 5 – 3 h after enzyme administration If the administered enzyme was not located within the β-cell lysosomes then the free" activity of the osmotically protected islet homogenate would be considerably augmented which was not the case (unpublished) The preserved activity of the enzyme after penetration into the β-cell and its possible implication in insulin releasing processes was further supported by the observation (paper 14) that pretreatment with acid amyloglucosidase 3 h before the experiment markedly augmented glibenclamide-induced insulin release

The half-life of the intravenously injected enzyme in blood (18-19 min) was unaltered whether the enzyme was injected into normal mice or into alloxan diabetic animals that had been diabetic for 6 months (paper 12) Moreover the liver glycogen depleting effect of the acid amyloglucosidase in alloxan diabetic mice that had been diabetic for about 2 months was as pronounced as in normal mice These observations showed that the ability of the enzyme to penetrate through the vascular walls and through the liver cell membrane was the same in controls and in animals that had been severely diabetic for a considerable time

B Endogenous activity of acid amyloglucosidase in pancreatic β-cells

To further elucidate the possible role of acid amyloglucosidase in insulin secreting processes an investigation was undertaken to determine acid amyloglucosidase activity in isolated pancreatic islets of the mouse (paper 13) It was observed that amyloglucosidase activity in collagenase-isolated pancreatic islets of the normal NMRI-mouse displayed a pH-optimum around pH 5 0 – 5 5 The enzyme could thus be characterized as an acid amyloglucosidase The activity of acid amyloglucosidase at pH 5 0 in islet tissue was twice that of liver and 10 times that of skeletal muscle The finding that the acid amyloglucosidase activity in islet tissue from alloxan diabetic mice was markedly reduced, to about 10 % of normal level, suggests that the activity is mainly localized in the β-cell. The hypothesis that the hydrolytic breakdown of β-cell glycogen plays a role for insulin releasing processes was strengthened by the observation (paper 13)

that the acid amyloglucosidase activity in islets of the insulin hyper-secreting obese mouse (AO-mouse) was more than twice that of its lean litter mates or the normal NMRI-mouse

C Interaction of monoamines with acid amyloglucosidase

It has been known for several years from in vitro experiments that certain glycoside hydrolases are inhibited by Tris (Larner and Gillespie 1956 Halvorsen and Ellias 1958 Dahlqvist 1961 Jørgensen 1963) There is some dispute whether the inhibitory effect of Tris is a function of its amino-group or its hydroxyl groups (cf Kelemen and Whelan 1965 Dahlqvist 1961) With regard to the purified fungal acid amyloglucosidase from Aspergillus niger it was thought probable that the inhibition by Tris at neutral pH is due to the amino-groups because both Tris and other amines such as cetylpyridinium chloride and protamine hydrochloride were found to be potent inhibitors at pH 7 0 (paper 4) As glibenclamide-induced insulin release was considerably enhanced by the previous treatment with purified acid amyloglucosidase (paper 14) whereas pretreatment with an amine precursor (L-5-HTP) or a monoamine oxidase inhibitor (paper 11) significantly inhibited glibenclamide--stimulated insulin release the question arose whether an interaction between monoamines and acid amyloglucosidase in the β-cell could possibly contribute to an explanation of the latter observation. It was observed (paper 14) that the marked enhancement of glibenclamide-induced insulin release after enzyme pretreatment was impaired after treatment with the monoamine precursors L-5-HTP and L-DOPA and also after treatment with a monoamine oxidase inhibitor The combined action of L-DOPA and a monoamine oxidase inhibitor caused the most marked inhibition, although it did not totally suppress the glibenclamide-induced insulin release The failure of the latter treatment to abolish glibenclamide--induced insulin release is in accordance with the observation that part of the insulin pool which can be released by the sulphonylurea compound is not affected by the amine (paper 11) As the amine is probably associated with the specific secretory granules (paper 10) it is tempting to speculate that the "amine-independent" insulin release is achieved through the alternative insulin secretion route which is presumed to by-pass the secretory granules (Like and Miki 1967 Renold 1970) Model experiments in the test tube (paper 14) showed that dopamine and 5-HT are capable of significantly inhibiting the action of the purified acid amyloglucosidase at neutral pH (pH 7 2) whereas no inhibition was recorded at pH 5 0 which suggests that the actual intracellular pH is of decisive importance for the inhibitory action of the amines Thus shifts in intracellular pH (alteration of lysosomal integrity?) might

regulate the inhibitory effect elicited by the intracellular amines through their action on acid amyloglucosidase

D The mechanism of action of sulphonylurea-induced insulin secretion
Lysosomes and insulin secretion

The amyloglucosidase activity in isolated pancreatic islets was found to display sedimentability (10 500 x g for 10 min) structure-linked latency and an acid pH-optimum (paper 13) (for definitions cf Vaes 1966) These properties are inherent in the concept of lysosomes and suggest a possible lysosomal localization of islet acid amyloglucosidase which points to an implication of lysosomes in insulin secreting processes Being a macromolecule the exogenous acid amyloglucosidase presumably enters the β-cell through endocytosis The endocytic vesicle would be incorporated into a lysosome and the enzyme activity retained The action of the endogenous enzyme or the exogenous enzyme which is taken up and stored by the cell could thus be expected to involve the lysosomes Pretreatment with the purified acid amyloglucosidase was found to markedly enhance sulphonylurea-induced insulin release Moreover it was observed that there was a dose dependent increase in "free" activity of the enzyme 2 min after glibenclamide injection (paper 14) The "free" activity of islet acid amyloglucosidase in control animals was found to be remarkably constant from experiment to experiment (papers 13 and 14); this increase was therefore interpreted as reflecting an enhancement of lysosomal permeability after sulphonylurea treatment The role of lysosomes in sulphonylurea-induced insulin release was further studied with the use of different steroids as lysosomal stabilizers and labilizers (cf Weissmann 1969) It was observed (paper 14) that glibenclamide-induced insulin release was enhanced after pretreatment with progesterone a lysosomal labilizer but diminished after pretreatment with dexamethasone a lysosomal stabilizer

Gylfe (1971) showed that glibenclamide added in vitro increases the release of particle-bound islet β-glucuronidase and acid phosphatase in crude lysosomal fractions from pancreatic islets of obese hyperglycaemic mice Hellman Idahl and Danielsson (1969) reported that the compound induced β-cell glycogenolysis Howell and Lacy (1969) showed that labelled glibenclamide penetrated into intact islet cells All this evidence suggests that sulphonylurea-stimulated insulin release is characterized by the following events in the β-cell Lysosomal activation - Increase of the "free activity of the lysosomal acid amyloglucosidase - Hydrolytic glycogen breakdown through acid amyloglucosidase yielding glucose - Insulin release

The possibility that the secretory granule itself is provided with intragranular lysosomal structures or lysosomal enzymes is unlikely because isolated β-granules are stable in the presence of sulphonylureas (Coore et al, 1969 Howell Young and Lacy 1969) which labilize islet lysosomes both in vitro (Gylfe 1971) and in vivo (paper 14) Moreover several physicochemical properties of insulin secretory granules clearly differ from those generally ascribed to lysosomes (Coore et al, 1969 Howell Young and Lacy 1969 Lambert et al, 1970 Beaufay 1969 Lacy 1969) An electron-microscope study failed to demonstrate acid phosphatase an enzyme predominantly present in lysosomes in the secretory granules of rabbit β-cells (Lazarus Volk and Barden 1966) A similar study of rat β-cells recently published (Orci et al 1971) however showed this enzyme to be present in a number of secretory granules This finding raises the question of whether a certain lysosome population may be closely associated with the secretory granules There are several morphological observations connecting the secretory granules of polypeptide producing endocrine cells with lysosomal autophagy" showing lysosomal granulolysis in the degradation of undischarged secretory products (Smith and Farquhar 1966 Orci et al 1968 Creutzfeldt et al, 1969 Boquist 1970) De Duve (1969) proposed that such processes should be designated crinophagy From the above-discussed data there seems to be good evidence that β-cell lysosomes are indeed implicated in insulin releasing processes and not only involved in the disposal of excess secretory products

VII. SUMMARY

The influence of extracellular monoamines β-cell monoamines and acid amyloglucosidase on insulin secretion was investigated in mice In addition, the insulin secretory capacity and the carbohydrate balance during the developmental and manifest stage of alloxan diabetes was studied in order to obtain models resembling various diabetic conditions Methods for glycogen determination purification of fungal acid amyloglucosidase and determination of acid amyloglucosidase in isolated pancreatic islets were developed Serial blood sampling in unanaesthetized mice was applied for the purpose of glucose determination.

The results obtained were interpreted as follows:

Acute diabetogenesis after alloxan is characterized by an initial hyperglycaemic phase due to marked liver glycogenolysis and cessation of insulin secretion. The ensuing hypoglycaemic phase is due to uncontrolled insulin leakage by the damaged β-cells Manifest alloxan diabetes is characterized by absence of or severely impaired insulin secretion, decreased levels of liver and muscle glycogen and high concentrations of blood glucose Diabetic animals in the β-cell regenerating phase are characterized by relatively high blood glucose levels and a significant number of functioning β-cells

Adrenaline has a dual action on insulin secretion analogous to its effect on the vascular system During basal conditions adrenaline will stimulate insulin secretion through its β-adrenergic properties whereas elevated levels of the amine have an inhibitory action through its α-adrenergic properties Intracellular monoamines in the β-cell are associated mainly with the insulin secretory granules They are able to inhibit insulin secreting processes possibly through the interaction of the monoamine with an enzyme acid amyloglucosidase shown in the present study to be present in the β-cell This hydrolytic enzyme displays sedimentability structure-linked latency and an acid pH-optimum These properties are interpreted as indicating a lysosomal localization of the enzyme Acid amyloglucosidase is implicated in insulin secreting processes through its ability to liberate glucose from β-cell glycogen. Its activity in isolated pancreatic islets from alloxan diabetic mice is about 10 % of that from normal mice On the other hand, in insulin hypersecreting obese mice the activity of the enzyme is more than twice that of normal mice

Highly purified fungal acid amyloglucosidase given to mice is taken up by the β-cell and stored in the lysosomes Administration of the enzyme to normal mice or alloxan diabetic mice in the β-cell regenerating phase results in an elevation of basal plasma insulin levels Moreover pretreatment of normal mice with the purified enzyme results in a marked enhancement of sulphonylurea-induced insulin release

A sulphonylurea compound augments the proportion of free" activity of islet acid amyloglucosidase after administration in vivo Stimulation of insulin release by a sulphonylurea compound is decreased by pretreatment with a lysosomal stabilizer but increased by a lysosomal labilizer

It is suggested that β-cell lysosomes are implicated in certain insulin releasing processes evoked through the following events in the β-cell: Lysosomal activation - Increase of the "free" activity of acid amyloglucosidase - Hydrolytic glycogen breakdown through acid amyloglucosidase yielding glucose - Insulin release

I wish to express my sincere gratitude to
Professor Stephen Thesleff head of this department who kindly placed laboratory facilities to my disposal and who has read and criticized the manuscript with great care to
Dr Claus Rerup who has been my teacher and under whose guidance it has been my privilege to work to
Professor Ragnar Ekholm and Drs Rolf Håkanson Lars Ericson, Kai Lindstrand Jan Börjeson Jan-Owe Josefsson and Christer Owman for fruitful co-operation and stimulating discussions to
Other colleagues in the Department of Pharmacology for stimulating discussions and encouraging interest to
Dr John Harris Department of Neurology University of Newcastle School of Medicine for careful reading and correction of my papers to
Mrs Lena Kvist, Miss Gertie Malmstein Miss Siw Erman Miss Monica Johansson Miss Anita Åkesson Miss Ann-Christin Helander Mrs. Karin Irenesson Miss Christina Lilja and Miss Margareta Lönnerberg for their skilful technical assistance and to
The Medical Faculty University of Lund the Swedish Diabetes Association the Swedish Society for Medical Research Vera och Carl J Michaelsens donationsfond" and Dr Martha Person Hennings donationsfond" for financial support of the investigations

IX. REFERENCES

ABRAMSON E A. and R A ARKY Role of beta-adrenergic receptors in counterregulation to insulin-induced hypoglycaemia Diabetes 1968 17 141 - 146
ÅKERBLOM, H K J M MARTIN and H E CINGOLANI Circulating glucose insulin, free fatty acids and acetone bodies in rats given propranolol Amer J Physiol 1969 217 1690 - 1693
ALLISON S P M.J CHAMBERLAIN J E MILLER R FERGUSON, A P GILLETT B V BEMAND and R A SAUNDERS Effects of propranolol on blood sugar insulin and free fatty acis. Diabetologia 1969 5 339 - 342
AURICCHIO F and V SICA. Dextran-gel filtration of rat liver α-glucosidases J. Chromatog. 1967 28 26 - 29

BAIRD C W and J BORNSTEIN Assay of insulin-like activity in the plasma of normal and diabetic and human subjects J Endocr 1959 19 74 - 80

BARKER S A and J G FLEETWOOD Studies on Aspergillus niger Part VIII The purification of glucamylase J Chem Soc 1957 4 4857 - 4864

BAUDHUIN P H.G HERS and H LOEB An electron microscopic and biochemical study of type II glycogenosis Lab, Invest. 1964 13 1139 - 1152

BEACH E F P J BRADSHAW and N R BLATHERWICK Alloxan diabetes in the albino rat as influenced by sex Amer J Physiol 1951 166 364 - 373

BEAUFAY H Methods for the isolation of lysosomes In Lysosomes in biology and pathology Vol 2 ed. J T Dingle and H B Fell North-Holland Publishing Company Amsterdam/London 1969 515 - 546

BERTLER Å. A CARLSSON and E ROSENGREN. A method for the fluorimetric determination of adrenaline and noradrenaline in tissues Acta physiol scand 1958 44 273 - 292

BOQUIST L Intracellular digestion and structural variations of secretory granules in pancreatic islet β-cells An ultrastructural study on diabetic and non-diabetic rodents Horm Metab Res 1970 2 166 - 171

BROLIN S E E BORGLUND and A. OHLSSON On the enzymatic activity of the pancreatic islets and acini in New Zealand obese mice In The structure and metabolism of the pancreatic islets ed. S E Brolin, B Hellman and H Knutson Pergamon Press Oxford 1964 289 - 294

BUCKLER J M H. A.M. BOLD M TABERNER and D R LONDON Modification of hormonal responses to arginine by α-adrenergic blockade Brit. Med. J, 1969 3 153 - 154

BUNNAG S C N E WARNER and S BUNNAG Effect of alloxan on the mouse pancreas during and after recovery from diabetes Diabetes 1967 16 83 - 89

BUSE M.G A.H JOHNSON D KUPERMINC and J BUSE Effect of α-adrenergic blockade on insulin secretion in man. Metabolism 1970 19 219 - 225

CARLSSON A. and B WALDECK A fluorimetric method for the determination of dopamine (3-hydroxytyramine) Acta physiol scand 1958 44 293 - 298

CARO L G and R P van TUBERGEN High-resolution autoradiography I. Methods J Cell Biol 1962 15 173 - 188

CARROLL, N V R W LONGELEY and J H. ROE The determination of glycogen in liver and muscle by use of anthrone reagent J biol Chem 1956 220 583 - 593

CEGRELL, L Monoamine-containing cells in the fetal and newborn guinea-pig pancreas Life Sci, 1967 6 1647 - 1652

CEGRELL, L The occurrence of biogenic monoamines in the mammalian endocrine pancreas Acta physiol scand 1968 Suppl 314

CEGRELL, L. B FALCK and B HELLMAN Monoaminergic mechanisms in the endocrine pancreas In The structure and metabolism of the pancreatic islets ed. S E Brolin, B Hellman and H Knutson Pergamon Press Oxford 1964 429 - 435

CERASI E S EFFENDIC and R. LUFT Role of adrenergic receptors in glucose-induced insulin secretion in man Lancet 1969 2 301 - 302

COLWELL, A.R and E M. BRIGHT The use of constant glucose injections for the study of induced variations in carbohydrate metabolism IV Suppression of glucose combustion by continuous prolonged epinephrin administration Amer J Physiol 1930 92 555 - 567

COORE H.G B HELLMAN E PIHL and I -B TÄLJEDAL Physicochemical characteristics of insulin secretion granules Biochem, J, 1969 111, 107 - 113

COORE H G and P J RANDLE Regulation of insulin secretion studied with pieces of rabbit pancreas incubated in vitro Biochem, J 1964 93 66 - 78

COHEN K L. and M.W BITENSKY Inhibitory effects of alloxan on mammalian adenyl cyclase J Pharmacol exp Ther 1969 169 80 - 86

CREUTZFELDT W C CREUTZFELDT H. FRERICHS E PERINGS and K. SICKINGER The morphological substrate of the inhibition of insulin secretion by diazoxide Horm Metab Res 1969 1 53 - 64

CROFFORD O B and C K DAVIS Jr Growth characteristics glucose tolerance and insulin sensitivity of New Zealand obese mice Metabolism, 1965 14 271 - 280

CURRY D L Factors affecting insulin secretion in vitro Amer, J, Clin Nutr, 1970 23 306 - 310

CUTHBERTSON W F J I.D FLEMING and M.S RICE Effect of glucamylase on tissue glycogen and tissue glucamylase in the rat. Biochem, J 1967 103 307 - 315

DAHLQVIST A. Determination of maltase and isomaltase activities with a glucose-oxidase reagent. Biochem, J, 1961 80 547 - 551

DE DUVE C The lysosome in retrospect In Lysosomes in biology and pathology Vol. 1 ed. J T Dingle and H B Fell. North-Holland Publishing Company Amsterdam/London. 1969 3 - 40

DUNN J C H L SHEEHAN and N G B MCLETCHIE Necrosis of islets of Langerhans produced experimentally Lancet 1943 1 484 – 487

FAIN J N R O SCOW and S S CHERNICK Effects of glucocorticoids on metabolism of adipose tissue in vitro, J biol Chem 1963 238 54 – 58

FALCK B and B HELLMAN Evidence for the presence of biogenic amines in pancreatic islets Experientia 1963 19 139 – 140

FALCK B N -Å HILLARP G THIEME and A TORP Fluorescence of catecholamines and related compounds condensed with formaldehyde J Histochem Cytochem 1962 10 348 – 354

FALCONI G and G L ROSSI Transauricular hypophysectomy in rats and mice Endocrinology 1964 74 301 – 303

FELDMAN, J M. and H E LEBOWITZ Serotonin inhibition of in vitro insulin release from golden hamster pancreas Endocrinology 1970 a 86 66 – 70

FELDMAN J M and H E LEBOWITZ Specificity of serotonin inhibition of insulin release from golden hamster pancreas Diabetes 1970 b 19 475 – 480

FELDMAN J M and H.E LEBOWITZ Mechanism of epinephrine and serotonin inhibition of insulin release in the golden hamster in vitro Diabetes 1970 c 19 480 – 487

FONG J F L SCHAFFER and P L KIRK. The ultramicrodetermination of glycogen in liver A comparison of the anthrone and reducing-sugar methods Arch, Biochem 1953 45 319 – 326

FREINKEL N M. MAGER and L VINNICK Cyclicity in the interrelationships between plasma insulin and glucose during starvation in normal young men J Lab clin Med 1968 71 171 – 178

FRENCH D and D W KNAPP The maltase of Clostridium Acetobutylicum, Its specificity range and mode of action J biol Chem 1950 187 463 – 471

FROHMAN L A The endocrine function of the pancreas Annu, Rev Physiol, 1969 31 353 – 382

FROHMAN L A Stimulation of insulin secretion in rats by pargyline and mebanazine Diabetes 1971 20 266 – 270

FROHMAN L.A E Z EZDINLI and R JAVID Effect of vagotomy and vagal stimulation on insulin secretion Diabetes 1967 16 443 – 448

GAGLIARDINO J J C F BELLONE I DORIA J J SANCHEZ and V PEREYRA Adrenergic regulation of basal serum glucose NEFA and insulin levels Horm Metab Res 1970 a 2 318 – 322

GAGLIARDINO J J R E HERNÁNDEZ R R RODRÍGUEZ and H C LAURI Stimulatory effect of nialamide on serum levels of insulin. Amer J Physiol 1970 b 219 314 – 317

GAGLIARDINO J J L. M. ZIEHER F C ITURRIZA, R E HERNÁNDEZ and R. R. RODRÍGUEZ Insulin release and glucose changes induced by serotonin Horm Metab Res 1971 3 145 - 150
GELOTTE B Separation of pancreatic enzymes by gel filtration Acta Chem Scand 1964 18 1283 - 1291
GENNSER, G I LUNDQUIST and E NILSSON Glycogenolytic activity in the liver of the human foetus Biol Neonate 1971 19 1 - 24
GERSHON M D. and L. L. ROSS Location of sites of 5-hydroxytryptamine storage and metabolism by radioautography J. Physiol 1966 186 477 - 492
GOETZ F C P M. BEIGELMAN and G W THORN A method of insulin bio-assay and its application to human plasma fractions Proc Soc exp Biol Med, 1954 86 484 - 486
GOOD C A. H. KRAMER and M SOMOGYI The determination of glycogen. J biol Chem 1933 100 485 - 491
GREENWOOD C T and E A MILNE Starch degrading and synthesizing enzymes A discussion of their properties and action pattern V Alpha amylases In Advanc Carbohydr Chem ed M L Wolfram and R S Tipson Academic Press New York/London 1968 23 205 - 230
GRODSKY G M and P H. FORSHAM Insulin and the pancreas Annu Rev Physiol 1966 28 347 - 380
GYLFE E Lysosomal activity and pancreatic β-cell function. Abstr. 7th Annu Meet Scand Soc Study Diabetes Sandefjord Norway 1971 p 21
HÅKANSON R. Radiometric micromethods for the study of some amino acid decarboxylases Acta pharmacol toxicol 1966 24 217 - 231
HÅKANSON R and CH. OWMAN Effect of denervation and enzyme inhibition on DOPA decarboxylase and monoamine oxidase activities of rat pineal gland J Neurochem 1965 12 417 - 429
HALVORSEN H. and L. ELLIAS The purification and properties of an α-glucosidase of Saccharomyces italicus Y 1225 Biochim biophys Acta, 1958 30 28 - 40
HEDING L A simplified insulin radioimmunoassay method. In Labelled proteins in tracer studies ed. L. Donato et al, Euratom Brussels 1966 345 - 350
HELLMAN, B and L Å IDAHL. On the functional significance of the pancreatic β-cell glycogen In The structure and metabolism of the pancreatic islets ed. S Falkmer B Hellman and I -B Täljedal Pergamon Press Oxford 1970 253 - 262
HELLMAN B L. Å IDAHL and Å DANIELSSON Adenosine triphosphate levels of mammalian pancreatic B cells after stimulation with glucose and hypoglycaemic sulfonylureas Diabetes, 1969 18 509 - 516

HERS H G α-Glucosidase deficiency in generalized glycogen-storage disease (Pompe s disease) Biochem J, 1963 86 11 - 16
HERTELENDY F K TAKAHASHI L.J MACHLIN and D M KIPNIS The effect of chronic adrenergic blockade on the inhibition by epinephrine of growth hormone and insulin release in sheep Horm Metab Res 1970 2 257 - 259
HIMMS-HAGEN J Sympathetic regulation of metabolism Pharmacol. Rev 1967 19 367 - 461
HIMMS-HAGEN J Adrenergic receptors for metabolic responses in adipose tissue Federation Proc 1970 29 1388 - 1401
HIRAI K and K GONDO Über DOPA-hyperglykämie Biochem Z, 1927 189 92 - 100
HOPKINS R H and D KULKA The glucamylase and debrancher of β diastaticus Arch. Biochem. Biophys 1957 69 45 - 55
HOUSSAY B A O ORÍAS and I. SARA. The mechanism of action of alloxan on blood sugar Science, 1945 102 p 197
HOWE R. and R G SHANKS Optical isomers of propranolol Nature 1966 210 1336 - 1338
HOWELL, S L and P E LACY Studies of the effect of HB 419 (glibenclamide) on isolated islets and granules Horm Metab Res 1969 1 Suppl pp 45 - 47
HOWELL, S L. and K W TAYLOR The acute pancreatic effect of alloxan in the rabbit J Endocr, 1967 37 421 - 427
HOWELL S L. D A YOUNG and P E LACY Isolation and properties of secretory granules from rat islets of Langerhans III Studies of the stability of the isolated beta granules J Cell Biol, 1969 41 167 - 176
HUG G and W K SCHUBERT Lysosomes in type II glycogenosis J Cell Biol, 1967 35 C1 - C6
HUGGETT A.S G and D A NIXON Use of glucose oxidase peroxidase and o-dianisidine in determination of blood and urinary glucose Lancet 1957 2 368 - 370
HUGHES H. L. L. WARE and F G YOUNG Diabetogenic action of alloxan Lancet 1944 1 148 - 150
HUIJING F A rapid enzymic method for glycogen estimation in very small tissue samples Clin. Chim Acta 1970 30 567 - 572
JAIM-ECHEVERRY G and L. M. ZIEHER. Electron microscopic cytochemistry of 5-hydroxytryptamine in the beta cells of guinea pig endocrine pancreas Endocrinology 1968 83 917 - 923
JØRGENSEN O B Barley malt α-glucosidase II Studies on the substrate specificity Acta Chem Scand 1963 17 2471 - 2478
KEILIN D and E F HARTREE Properties of glucose oxidase (notatin) Biochem J 1948 42 221 - 229

KELEMEN M V and W J WHELAN Selective inhibition of glucosidases by polyols Abstr 2nd Meet Fed Europ Biochem Soc Vienna A25 1965 18 - 19

KERR R W F C CLEVELAND and W J KATZBECK The action of amylo-glucosidase on amylose and amylopectin J Amer Chem Soc 1951 73 3916 - 3921

KRIS A.O R E MILLER F E WHERRY and J W MASON Inhibition of insulin secretion by infused epinephrine in rhesus monkeys Endocrinology 1966 8 87 - 97

LACY P E The pancreatic beta cell Structure and function New Engl J Med, 1967 276 187 - 191

LACY P E Beta cell secretion - from the standpoint of a pathobiologist Diabetes, 1970 19 895 - 905

LAMBERT A.E Y KANAZAWA L. ORCI and G M GRODSKY Properties of isolated β-granules in suspension. In The structure and metabolism of the pancreatic islets ed S Falkmer B Hellman and I -B Täljedal Pergamon Press Oxford 1970 397 - 40

LARNER J and R.E GILLESPIE Gastrointestinal digestion of starch II. Properties of intestinal carbohydrases J biol Chem 19 6 223 709 - 726

LAZARUS, S S B W VOLK and H BARDEN Localization of acid phosphatase activity and secretion mechanism in rabbit pancreatic B-cells J Histochem Cytochem 1966 14 233 - 246

LEONARDS J R. and B R. LANDAU A study on the equivalence of metabolic patterns in rat adipose tissue Insulin versus glucose concentration Arch Biochem Biophys 1960 91 194 - 200

LERNMARK Å The significance of 5-hydroxytryptamine for insulin secretion in the mouse Horm Metab Res 1971 3 305 - 309

LEVINE R Mechanisms of insulin secretion New Engl J Med 1970 283 522 - 526

LIKE A.A and E MIKI Diabetic syndrome in sand rats IV Morphologic changes in islet tissue Diabetologia, 1967 3 143 - 166

LOUBATIÈRES A. M M MARIANI J CHAPAL J TAYLOR M.H. HOUAREAU et A M RONDOT Action nocive de l adrénaline pour la structure histologique des îlots de Langerhans du pancréas Action protectrice de la dihydroergotamine Diabetologia, 1965 1 13 - 20

LOWRY O H. N J ROSEBROUGH A L. FARR and R.J RANDALL Protein measurement with the Folin phenol reagent J. biol. Chem 1951 193, 265 - 275

LUCY J A Lysosomal membranes In Lysosomes in biology and pathology, Vol 2 ed. J T Dingle and H B Fell North-Holland Publishing Company Amsterdam/London. 1969 313 - 341

LUNDQUIST I On the significance of serum dilution and cortisol antagonism in the rat fat pad bioassay of insulin Acta endocr 1968 58 11 - 26

LUNDQUIST I Glucose utilization and adrenergic receptors in muscle and liver tissue Acta pharmacol toxicol 1971 29 Suppl 4 p 34

LUNDQUIST I R EKHOLM and L. E ERICSON Influence of 5-hydroxytryptamine on insulin secretion Acta endocr, 1971 67 Suppl 155 p 188

LUNDQUIST I and C RERUP Blood glucose level in mice 3 On the nature of corticotrophin induced hypoglycaemia. Acta endocr, 1967 56 713 - 725

LYNGSØE J Determination of the insulin-like activity in serum using rat epididymal adipose tissue Scand J Lab clin Invest 1961 13 628 - 636

MARKS V An improved glucose-oxidase method for determining blood C S F and urinary glucose levels Clin Chim Acta 1959 4 395 - 401

MARSHALL J J and P M. TAYLOR Acid α-glucosidases from plant sources Biochem Biophys Res Comm 1971 42 173 - 179

MATSCHINSKY F M and J E ELLERMAN Metabolism of glucose in the islets of Langerhans J biol Chem 1968 243 2730 - 2736

MAUNSBACH A. B Absorption of ^{125}I-labelled homologous albumin by rat kidney proximal tubule cells A study of microperfused single proximal tubules by electron microscopic autoradiography and histochemistry J Ultrastruct Res 1966 15 197 - 241

MAYHEW D A. P H WRIGHT and J ASHMORE Regulation of insulin secretion. Pharmacol Rev 1969 21 183 - 212

MIDDLETON J E and W J GRIFFITHS Rapid colorimetric micro--method for estimating glucose in blood and C S F using glucose oxidase Brit, Med J, 1957 2 1525 - 1527

MILLER R E and J S SOELDNER Suppression of portal venous insulin concentration by epinephrine in the conscious monkey Diabetologia 1969 5 179 - 182

MOSKALEWSKI S Isolation and culture of the islets of Langerhans of the guinea pig Gen Comp Endocr 1965 5 342 - 353

NICKERSON M. Drugs inhibiting adrenergic nerves and structures innervated by them In The Pharmacological Basis of Therapeutics ed. L S Goodman and A Gilman Macmillan New York 1965 546 - 577

ORCI, L. A. JUNOD R PICTET A E RENOLD and C ROUILLER Granulolysis in A cells of endocrine pancreas in spontaneous and experimental diabetes in animals J Cell Biol, 1968 38 462 - 466

ORCI L. W STAUFFACHER D BEAVEN A E LAMBERT A.E RENOLD and C ROUILLER. Ultrastructural events associated with the action of tolbutamide and glybenclamide on pancreatic B-cells in vivo and in vitro Acta diabet lat 1969 6 Suppl 1 271 - 274

ORCI, L. W STAUFFACHER C RUFENER A E LAMBERT C ROUILLER and A.E RENOLD Acid phosphatase activity in secretory granules of pancreatic beta cells of normal rats Diabetes, 1971 20 385 - 388

PAZUR, J H. and T ANDO The action of an amyloglucosidase of Aspergillus niger on starch and malto-oligosaccharides J Biol. Chem. 1959 234 1966 - 1970

PAZUR, J H. and T ANDO The hydrolysis of glucosyl oligosaccharides with α-D-(1 - 4) and α-D-(1 - 6) bonds by fungal amyloglucosidase J. Biol Chem, 1960 235 297 - 302

PAZUR, J H. and K KLEPPE The hydrolysis of α-D-glucosides by amyloglucosidase from Aspergillus niger J Biol Chem 1962 237 1002 - 1006

PAZUR, J H. and S OKADA. Properties of the glucoamylase from Rhizopus delemar Carbohydr. Res. 1967 4 371 - 379

PETTIT A. Procédé simple pour préserver du sang chez les petits rongeurs C. R. Soc. Biol. (Paris) 1913 74 11 - 12

PHILLIPS L.L. and M L. CALDWELL. A study of the purification and properties of a glucose-forming amylase from Rhizopus delemar gluc amylase J. Amer Chem. Soc. 1951 73 [illegible] - 3563

PINTER, E J C J PATTEE G PETERITT and J M CLEGHORN Metabolic effects of autonomic blockade Lancet, 1967 2 101 - 10[illegible]

POOL, E.L. and L.A. UNDERKOFLER Fungal saccharifying agents Amylolytic factors of bran culture and submerged culture J. Agr. Food. Chem. 1953 1 87 - 90

PORTE D Jr A receptor mechanism for the inhibition of insulin release by epinephrine in man J. clin Invest. 1967 46 86 - 94

PORTE D Jr Sympathetic regulation of insulin secretion Arch. Intern. Med 1969 123 252 - 260

PORTE, D Jr and J D BAGDADE Human insulin secretion An integrated approach Ann. Rev. Med 1970 21 219 - 240

PORTE D Jr A. GRABER, T KUZUYA and R.H. WILLIAMS The effect of epinephrine on immunoreactive insulin levels in man J. clin. Invest. 1966 45 228 - 236

PORTE D Jr and R. H. WILLIAMS Inhibition of insulin release by norepinephrine in man Science, 1966 152 1248 - 1250

REESE E T A.H MAGUIRE and F W PARRISH Glucosidases and exo-glucanases Can. J. Biochem, 1968 46 25 - 34

RENOLD A.E Insulin biosynthesis and secretion - a still unsettled topic New. Engl J. Med 1970 282 173 - 182

RERUP C Spontaneous remission of alloxan diabetes in mice Diabetologia 1968 4 p 312

RERUP C Drugs producing diabetes through damage of the insulin secreting cells Pharmacol Rev 1970 22 485 – 518

RITZEN M L HAMMARSTRÖM and S ULLBERG Autoradiographic distribution of 5-hydroxytryptamine and 5-hydroxytryptophan in the mouse Biochem Pharmacol 1965 14 313 – 321

ROSENFELD E L I A POPOVA and V S ORLOVA Influence of adrenaline on the splitting of glycogen and maltose by γ-amylase of liver heart and skeletal muscles of the rat. Bull Soc Chim Biol, 1970 52 1111 – 1116

SAMOLS E G MARRI and V MARKS Interrelationship of glucagon insulin and glucose Diabetes, 1966 15 855 – 866

SEIFTER S S DAYTON B NOVIC and E MUNTWYLER The estimation of glycogen with the anthrone reagent. Arch Biochem 1950 25 191 – 200

SMITH, R E and M G FARQUHAR Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland J. Cell Biol 1966 31 319 – 347

SMITH, E E P M TAYLOR and W J WHELAN Enzymic processes in glycogen metabolism In Carbohydrate Metabolism and Its Disorders Vol I ed F Dickens P J Randle and W J Whelan Academic Press London/New York 1968 89 – 138

SUTHERLAND E W and G A. ROBISON The role of cyclic AMP in the control of carbohydrate metabolism Diabetes, 1969 18 797 – 819

TAKAHASHI M and T SHIMOMURA Biochemical studies on α-glucosidase from buckwheat (Fagopyrum esculentum mönch) Part I Screening of α-glucosidase from plant sources and changes of α-glucosidase activity during the germination stages of buckwheat Agr Biol Chem 1968 32 923 – 928

TELIB M S RAPTIS K E SCHRÖDER and E F PFEIFFER Serotonin and insulin release in vitro, Diabetologia 1968 4 253 – 256

TJÄLVE H Catechol- and indolamines in some endocrine cell systems *Acta physiol scand 1971 Suppl 360*

TORESON W E Glycogen infiltration (so-called hydropic degeneration) in the pancreas in human and experimental diabetes mellitus Amer J Path 1951 27 327 – 336

TSUJISAKA Y FUKOMOTO and T YAMAMOTO Specificity of crystalline saccharogenic amylase of moulds Nature 1958 181, 770 – 771

VAES G Subcellular localization of glycosidases in lysosomes In Methods in Enzymology Vol VIII Complex Carbohydrates ed. E F Neufeld and V Ginsburg Academic Press London/New York 1966 509 – 514

WERRBACH, J H. C C GALE C J GOODNER and M.J CONWAY Effects of autonomic blocking agents on growth hormone insulin free fatty acids and glucose in baboons Endocrinology 1970 86 77 – 82

WONG K K. S SYMCHOWICZ M S. STAUB and I I A TABACHNICK. The in vitro effect of catecholamines diazoxide and theophylline on insulin release Life Sci. 1967 6 2285 – 2291

YALOW R.S and S A BERSON. Immunoassay of endogenous plasma insulin in man J clin Invest 1960 39 1157 – 1175

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 373

LASTING SELECTIVE DEPLETION OF BRAIN SEROTONIN BY 5,6-DIHYDROXYTRYPTAMINE

BY

H. G. BAUMGARTEN, A. BJÖRKLUND, L. LACHENMAYER,
A. NOBIN and U. STENEVI

LUND 1971

STUDENTLITTERATUR
LUND SWEDEN

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 373

LONG-LASTING SELECTIVE DEPLETION OF BRAIN SEROTONIN BY 5,6-DIHYDROXYTRYPTAMINE

BY

H. G. BAUMGARTEN, A. BJÖRKLUND, L. LACHENMAYER, A. NOBIN and U. STENEVI

LUND 1971

STUDENTLITTERATUR
LUND SWEDEN

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 373

FROM THE DEPARTMENT OF ANATOMY AND NEUROANATOMY UNIVERSITY OF HAMBURG W GERMANY AND DEPARTMENT OF HISTOLOGY UNIVERSITY OF LUND SWEDEN

LONG LASTING SELECTIVE DEPLETION OF BRAIN SEROTONIN BY 5,6-DIHYDROXYTRYPTAMINE

BY

H. G BAUMGARTEN A. BJÖRKLUND L. LACHENMAYER,

A. NOBIN and U STENEVI

Abbreviations used. 5,6-DHT 5 6-dihydroxytryptamine 5-HT 5-hydroxytryptamine 6-OH DA, 6-hydroxydopamine CA, catecholamines; NA, noradrenaline, DA, dopamine.

SUMMARY

One dose of 25—75 μg of 5,6-dihydroxytryptamine (5,6-DHT) — a strongly reducing congener of serotonin — injected into the lateral ventricle caused a marked depletion of rat brain serotonin, which persisted up to the 30th day the longest post injection time studied. After a 75 μg injection, the long term depletion was highest, about 85 % in the spinal cord and lowest, about 20 % in the pons/medulla oblongata, whereas remaining brain regions — mesencephalon, hypothalamus, septum, striatum, and remaining telencephalic structures — showed approximately 40 to 60 % reduction in serotonin concentration. After the highest dose of 5,6-DHT 75 μg, there was also a rapid initial reduction in whole brain catecholamine levels, which was most marked, about 40 % for dopamine. The catecholamine concentrations, however returned to normal between the second and fourth day after injection. The longlasting depletion of brain serotonin by 5,6-DHT resembles the effect of 6-hydroxydopamine on brain catecholamines. These chemical observations and preliminary electron microscopical and fluorescence microscopical findings indicate that 5,6-DHT induces a chemical degeneration of central monoamine nerve terminals, which appears to be selective for serotonin neurons within the dose range used.

INTRODUCTION

When the remarkable, acute, and selective degenerative effect of 6-hydroxydopamine (6-OH DA) on peripheral sympathetic nerve terminals was demonstrated (Tranzer and Thoenen 1967 1968 Thoenen and Tranzer 1968 Malmfors and Sachs 1968) the concept of selective chemical denervation was introduced in neurobiological research. Also in brain, 6-OH DA, when injected in the ventricles or into the brain parenchyma, was shown to induce a long lasting depletion of cerebral catecholamines (Ungerstedt 1968, 1971 a, Uretsky and Iversen 1970) accompanied by a marked reduction in catecholamine uptake and in tyrosine hydroxylase activity (Breese and Traylor 1970, 1971 Iversen and Uretsky 1971) and by electron microscopic evidence for terminal degeneration (Bloom et al. 1969) These findings provide strong evidence for a cytotoxic effect of 6-OH DA also on brain catecholamine neurons. Within a properly selected dose range, this effect appears to be remarkably selective, and the brain serotonin neurons seems to be unaffected (Bloom et al. 1969 Ungerstedt 1971 a, Hökfelt and Ungerstedt 1971)

In such a complex and morphologically intricate structure as the brain, the selective chemical denervation is a most attractive approach to neuroanatomical and functional problems, particularly when dealing with such widespread and diffusely organized systems as the monoamine neuron systems

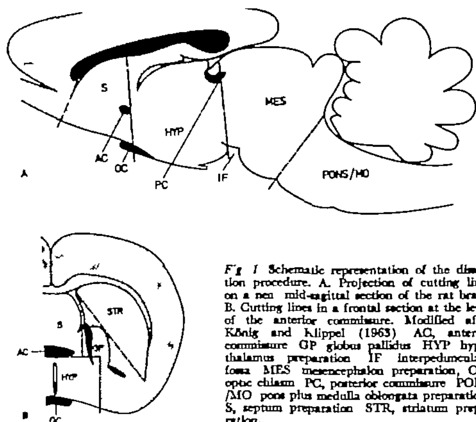


Fig. 1. Schematic representation of the dissection procedure. A. Projection of cutting lines on a para mid-sagittal section of the rat brain. B. Cutting lines in a frontal section at the level of the anterior commissure. Modified after König and Klippel (1963). AC, anterior commissure. GP, globus pallidus. HYP, hypothalamus preparation. IF, interpedunculary fossa. MES, mesencephalon preparation. OC, optic chiasm. PC, posterior commissure. PONS/MO, pons plus medulla oblongata preparation. S, septum preparation. STR, striatum preparation.

(cf. Ungerstedt 1971 b). It is obvious that chemical denervation offers possibilities for overcoming the drawbacks inherent in denervation through mechanical or electrolytical lesions, which in brain inevitably involves a number of different neuron systems and non-neuronal tissue elements. In the present paper we have studied the possibilities for a selective chemical destruction of brain serotonin neurons through a strongly reducing congener of serotonin, 5,6-dihydroxytryptamine (5,6-DHT).

MATERIAL AND METHODS

Adult (200—250 g) male and female albino rats (Wistar strain, AF Han) were used. The animals were pretreated under light ether anaesthesia with a single intraventricular injection of 25, 50 or 75 μg 5,6-DHT (creatininsulfate-H_2O-complex) calculated as the free base (for synthesis, see Schlossberger and Kuch 1960). The compound was chromatographically homogeneous. 5,6-DHT was dissolved in dilute mammalian Ringer (1/3 distilled water, 2/3 Ringer) containing 0.1 mg/ml ascorbic acid. 20 μl was injected into the left lateral ventricle via a Hamilton microliter syringe as devised by Noble, Wurtman and Axelrod (1967).

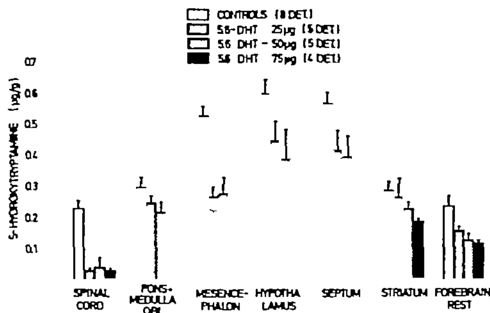
The animals were killed by decapitation 1 3 6 or 12 hours, or 1 2, 4 5 10 16 or 30 days after administration of the drug The brains were quickly removed from the skull, the olfactory bulbs removed, and the brains dissected for 5-HT estimates into the following six parts septum, striatum, hypothalamus, remaining forebrain, mesencephalon, and pons/medulla oblongata as shown in Fig 1 the cerebellum and pineal gland were discarded. The spinal cord dissections included the entire cord comprising cervical thoracal, and lumbar segments. The specimens were quickly frozen in liquid nitrogen and stored at this temperature until analysis.

5-hydroxytryptamine (5-HT) was estimated spectrofluorimetrically according to the method of Bertler (1961) Noradrenaline (NA) and dopamine (DA) were determined on whole brains (olfactory bulbs and pineal glands removed) according to Bertler Carlsson, Rosengren and Waldeck (1958) as modified by Häggendal (1963) 5,6-DHT was passed through the analytical procedures used for the 5-HT and NA/DA assays and found not to interfere in any respect with the determinations. The recovery in the 5-HT determinations averaged 86 %. The values were not corrected for recovery

For the 5-HT determinations, regions from 5 animals were pooled for each sample and the following number of samples were assayed untreated control rats 8 samples 1 3 and 12 hrs, and 4 10, 16 and 30 days after 75 μg 5,6-DHT 4 samples for each point 1 day after 75 μg 5,6-DHT 5 samples 2 days after 5 6-DHT 3 samples 10 days after 25 or 50 μg 5 6-DHT 5 samples for each point. For the whole-brain determinations of NA and DA one brain was used for each sample, and the following number of samples were assayed untreated controls 5 samples 1 3 and 12 hrs, and 1 2, 4 and 30 days after 75 μg 5 6-DHT 4 samples for each point 10 days after 75 μg 5 6-DHT 6 samples 10 days after 25 or 50 μg 5,6-DHT 3 and 5 samples respectively

RESULTS

The first set of experiments established that a maximum depletion of brain serotonin in the rat was accomplished by 10 days after a single intraventricular injection of 5 6-DHT This post injection time was therefore chosen for a comparison of the effect of different graded doses of 5,6-DHT (25 50 75 μg) on the 5-HT concentration in seven individual brain regions. Fig 2 shows that all doses tested caused a highly significant ($p<0.001$) drop of 5-HT in spinal cord mesencephalon, hypothalamus, septum, and the "forebrain rest" preparation, whereas the reduction was less in the pons/medulla oblongata ($0.05>p>0.001$) In striatum, the lowest 5,6-DHT dose had no significant effect on the 5-HT level, and only after 50 or 75 μg a significant reduction in 5-HT was obtained. A dose-dependent, graded reduction of 5-HT was found in the hypothalamus, septum, striatum, and forebrain rest" sample. In contrast to this, the extent of depletion of 5-HT was the same with 25 50 or



F g Effect of three different doses of 5 6-DHT on the 5-HT concentration in different brain regions, 10 days after intraventricular injection. Preparations from five rats were pooled for each determination. The bars give means ± S. D. of four or five determina tions. Differences from control value:
*=0.05<p<0.01 **=0.01<p<0 001 **=p<0.001 Student's t-test.

75 µg 5 6-DHT in the spinal cord pons/medulla oblongata, and mesencephalon (Fig 2)

The percentage depletion of 5-HT for a given dose of 5,6-DHT varied considerably in different brain regions. It was greatest in the spinal cord (about 85 % depletion) and least in the striatum and pons/medulla oblongata (10—40 %) whereas the reduction in the other regions was approximately 50—60 % (Figs. 2 and 4)

Ten days after 25—75 µg 5 6-DHT no significant alteration in whole brain DA concentration was found, whereas the NA concentration was significantly increased (0.01>p>0.001) (Fig 3)

The time course of the effect of a single intraventricular injection of 75 µg 5 6-DHT on the 5-HT concentration was analysed in the seven brain regions dissected according to the description given in Fig 1 and the effect of 5,6-DHT on the NA and DA concentrations was investigated on whole brain specimens. As seen from Fig 4 the reduction of 5-HT in the individual brain sections is long lasting and persists up to 30 days, the largest survival time followed up so far A more detailed analysis of the time course of effects of 5,6-DHT on the 5-HT content in the different brain regions reveals that three different patterns of 5-HT depletion and recovery can be distinguished.

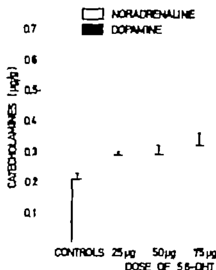


Fig 3 Effect of three different doses of 5,6-DHT on whole brain catecholamine concentrations, 10 days after intraventricular injection. The bars give means ± S.D of three to six determinations (see Material and Methods)

1 A rapid initial and pronounced fall of 5-HT which reaches a maximum at about 12 hrs after injection is found in the septum, striatum, hypothalamus, and mesencephalon (Fig 4 B and C) The 5-HT levels then remain low up to at least the 16th day between the 16th and the 30th day there is a slight but significant recovery in hypothalamus (difference between the 16- and 30-day values $0.01 > p > 0.001$) mesencephalon ($p < 0.001$) and septum ($0.05 > p > 0.01$)

2. In the pons/medulla oblongata preparation a clear drop of 5-HT is observed which is rapid in onset and fully developed at 12 hrs after the 5 6-DHT injection, but a marked, although incomplete, recovery of the 5-HT values occurs during the next four days. This increase in 5-HT between 12 hrs and 4 days is statistically significant ($0.01 > p > 0.001$)

3 A more retarded and gradual decline of the 5-HT concentration, which reaches maximum extent only at four days after the drug administration, is characteristic for the spinal cord and forebrain rest preparation. The 5-HT values then remain low although there is a slight, but not statistically significant increase in the 5-HT values of the forebrain preparation between the 16th and 30th day

The time course patterns of the effect of a single intraventricular injection of 75 μg 5 6-DHT upon whole brain NA and DA concentrations differ from those described for 5-HT Fig 5 shows that a rapid but moderate displacement of DA occurs in the brain, which is complete after 12 hrs and recovers

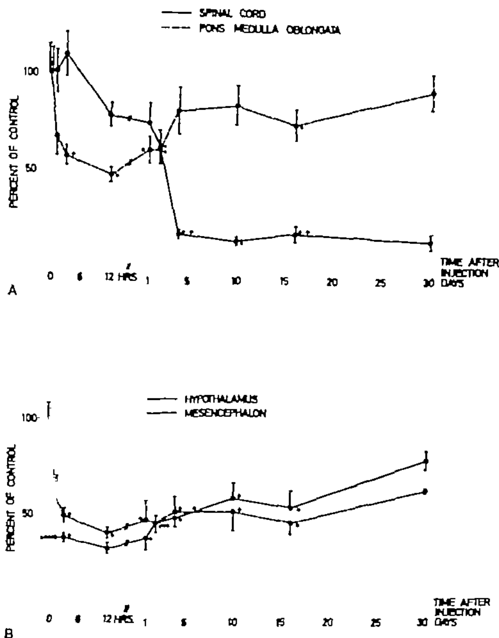
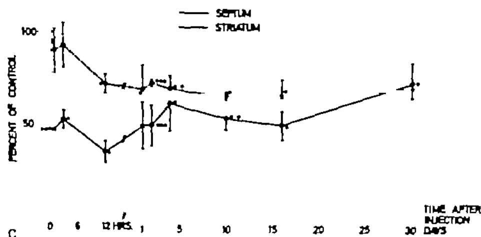


Fig. 4 A—D. Time course of effect of one single intraventricular injection of 75 µg 5,6-DHT on the 5-HT concentration in seven brain regions. Preparations from five rats were pooled for each determination. Each value is the mean ± S.D of three to eight determinations (see *Material and Methods for details*) Differences from control values *=0.05<p<0.01 **=0.01<p<0.001 **=p<0.001 Student' t-test.

gradually during the next few days. Near normal DA values (not statistically different from control values) were regained at four days. The whole brain noradrenaline concentration exhibited a rapid fall and rise during the first few hours after 5,6-DHT injection, followed by a gradual marked increase during the next 10 days (50 % increase above normal) Near normal values were restored at 30 days.



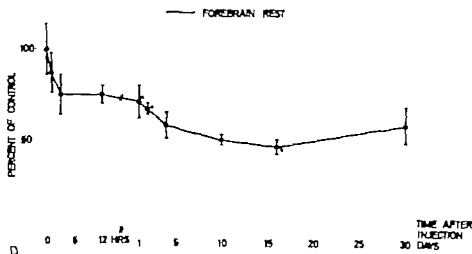


Fig 4 A and D: For legend, see opposite page.

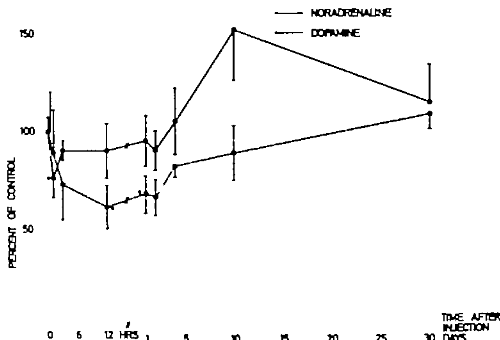


Fig 5 Time course of effect of one single intraventricular injection of 75 μg 5 6-DHT on whole brain NA and DA concentrations. Each point gives the mean ± S. D of four to six determinations. Differences from control values
*=0.05<p<0.01; **=0.01<p<0.001; ***=p<0.001 Student's t-test.

DISCUSSION

The results demonstrate that intraventricularly administered 5 6-DHT produces a marked and long lasting reduction in brain serotonin, similar to that caused on brain CA neurons by 6-OH DA. This parallelism suggests a similar mechanism of action for both drugs on CNS monoamine neurons. In fact preliminary fluorescence microscopical findings in 5,6-DHT treated animals (Baumgarten and Lachenmayer 1972, Baumgarten, Lachenmayer and Schlossberger 1972, Baumgarten, Björklund Holstein and Nobin 1972) have demonstrated a loss of indolamine terminals and a characteristic accumulation of the transmitter in preterminal axon bundles, in association with ultrastructural evidence of terminal degeneration. Taken together these data provide evidence that the reduction in brain serotonin by 5 6-DHT is the result of a chemical destruction of serotonin-containing terminals and, possibly axons. This destructive effect appears to be selective for serotonin neurons when the drug is administered in doses not higher than 75 μg.

The time-course of depletion of 5-HT was found to differ in different brain regions. When analysing the chemical data, it became obvious that we are dealing with three main types of time course patterns of 5 6-DHT effect.

1 In septum, striatum, hypothalamus, and mesencephalon, a maximum depletion is reached within 12 hrs, and the 5-HT levels remain low up to at least the 16th day This time course is similar to that reported by Uretsky and Iversen (1970) and Bell Iversen and Uretsky (1970) for noradrenaline in whole brain and hypothalamus, respectively after intraventricular injections of 6-OH DA. With 6-OH DA, there is strong evidence of a rapid depletion of noradrenaline accompanied by an acute degeneration of noradrenergic terminals localized near the ventricles (Bell Iversen and Uretsky 1970, Iversen and Uretsky 1971 Ungerstedt 1971 a) indicating that this type of time course pattern seen also with 5,6-DHT in septum, striatum, hypothalamus, and mesencephalon, reflects a direct, rapid terminal degeneration.

2. In pons/medulla oblongata 5 6-DHT causes an initial, rapid and marked 5-HT reduction, which reaches its maximum after 5 to 12 hours, but the 5-HT level recovers partially during the next four days. This can be explained in view of the fact that this brain region possesses major indolamine-containing cell groups, some of which occupy areas close to the fourth ventricle. It can thus be presumed that 5,6-DHT might have directly affected or temporarily damaged the cell bodies, reflected by a temporary depletion of their 5-HT content. An alternative explanation for the early partial recovery in this region could well be the heavy accumulation of transmitter in the ascending and descending 5-HT-containing axon bundles which develops during the first four days after treatment and persists up to at least the 16th day (Baumgarten Lachenmayer and Schlossberger 1972, Baumgarten, Björklund, Lachenmayer Nobin and Stenevi, in preparation)

3 In spinal cord and — although less pronounced — in the forebrain rest" preparation (comprising cortical structures, subcortical regions, such as, e.g., the amygdala and globus pallidus diencephalic centres such as, e.g thalamus, epithalamus, and the geniculate bodies) the loss of serotonin is considerably more retarded, showing a slow gradual decline and reaching maximum by four days. This retarded transmitter depletion could be due to an anterograde degeneration of nerve terminals after damage to preterminal axons or pericarya. Thus, the efficient destruction of axon bundles or cell groups lying close to the cerebrospinal fluid spaces would lead to a subsequent efficient degeneration also of their terminal areas. After mechanical lesions, such anterograde degeneration will result in such a slow and protracted depletion of the transmitter maximum depletion being reached after approximately 4 to 5 days (Moore and Heller 1967 see Moore 1970) In case of spinal cord, the slow depletion might thus well be explained by the very superficial localization of the descending preterminal serotonin axons in the white matter of the cord (see Dahlström and Fuxe 1964) thus being very close to the surrounding cerebrospinal fluid. This anatomical arrangement of the spinal cord innervation could also explain why the very marked reduction in spinal cord 5-HT was seen already with the lowest dose, 25 μg Effects probably

ACTA PHYSIOLOGICA
SUPPLEMENTUM 374

The Functional Ultrastructure of the Barrier Computer Analysis of Data from Lymph Experiments using Theoretical

BY

G ARTURSON T GROTH and G GROTTE

Stockholm 1972



To Olaus Rudbeck,
discoverer of the lymphatic circulation,
professor of medicin, University of Uppsala 1658—1692

Acta physiologica scandinavica supplementum 374

From the Burn Center and Department of Pediatric Surgery University Hospital nd the Institute of Physics, University of Uppsala, Sweden

The Functional Ultrastructure of the Blood Lymph Barrier Computer Analysis of Data from Dog Heart Lymph Experiments using Theoretical Models

By

G Arturson T Groth and G Grotte

Abstract

A generalized two-pore model including differences in permeability surface area nd pressure conditions along the capillary was formulated and certai simplifications were tested with respect to their influence on the estimated parameters. A simplified version of the model was tested for compatibility and parameter confidence against series of macromolecular sieving data obtained from Starling dog heart-lung preparations. In these experiments heart-lymph was collected during steady state conditions and the venous pressure was changed.

During normal" conditions the blood lymph barrier of the dog heart-lung preparations could be adequately described by an equivalent two-pore membrane with small pores of radius 35—60 Å, and an additional set of larger pores of radius 120—160 Å, with the density of one large pore per 10,000 small pores. The mean transcapillary pressure difference was calculated to be less than 1 cm H_2O

During elevated venous pressure the effective surface area for fluid exchange was found to decrease to 1/2—1/3 of the normal value due to pronounced decrease in small pore radius. The concomitant increase in large pore radius caused increased flow of fluid through the large pores. These results, in addition to the corresponding pressure perturbation data have given some support to the pressure compensatory mechanisms suggested by Wiederhielm (1968)

The hypothesis that the transport of macromolecules takes place by way of cytopempsis was shown to be tenable if the ad hoc assumption was made that the vesicle size or density is pressure dependent.

Introduction

The transfer of fluid across the capillary endothelium was studied by Starling (1896) and considered to be a passive process the rate and direction being dependent on the algebraic difference between hydrostatic and colloid osmotic pressures acting across the endothelial barrier This barrier was looked upon as a filter permeable to small, lipid-insoluble molecules, but impermeable to larger ones. Experimental work by Landis (1927 1932, 1946 1963) lent support to the Starling concept. Somewhat later Pappenheimer and coworkers (1948 1951 1953) could show that diffusion is the basis for the exchange of small, lipid-insoluble molecules between plasma and tissue. They developed the pore theory of capillary permeability i.e. that the transport of fluid and small lipid-insoluble molecules could largely be accounted for by the

assumption of uniform cylindrical pores of effective pore radius 40—50 Å, which occupy less than 0 1 % of the capillary surface Since such small pores restrict the penetration of large molecules, the observed slow passage of molecules larger than the small pores could be accounted for either by passage through a sparser set of bigger pores, about 250 Å in radius, as postulated by Grotte 1956 (cf. Mayerson *et al.* 1960) or by a vesicular transport system (Palade, 1956 Mayerson *et al* 1960 cp also Renkin, 1964)

Ultramicroscopic investigations of the porosity of capillaries have not been entirely reconcilable with the physiological data (Majno 1965 Luft 1965 1966 Florey 1966 Cotran, 1967) However the endothelial cell junctions with gaps of about 40 Å in width between maculae occludentes are most likely the morphological equivalent of the small pore system proposed for the passage of small lipid-insoluble molecules across the endothelium (Karnovsky 1967) Vesicular transport as the basis for the rapid transcapillary exchange of *small* molecules and fluid has been questioned on both morphological (Florey 1966 Fawcett, 1963 1965) and physiological (Renkin, 1964) grounds, as being insufficient in magnitude It has, however been suggested that vesicular transport may be responsible for the transport of *large* molecules (Mayer son *et al* 1960 Renkin, 1964) and that it may correspond to the large pore system postulated by Grotte (1956)

The hydrostatic pressure drop from arterial to venous end of the capillary bed was considered in a two-pore model by Winne (1965) which was thus more general than the model by Pappenheimer *et al.* However this model did not take into account differences in surface area (Wiedeman 1967) and permeability characteristics along the capillary which have been found to be of great importance for the understanding of capillary exchange (Wiederhielm, 1968) It was therefore felt necessary to extend the two pore model by Winne to include differences in porosity and surface area along the capillary and to estimate the influence of previously untested assumptions about these factors on calculated pore radii, pore density ratios and pressure para meters (Part I)

Part II of the present investigation presents data from a Starling dog heart lung preparation in which a lymphatic trunc, draining the heart muscle of mainly the left ventricle, was cannulated The micro-vascular permeability of the heart muscle was investigated by using dextran as a test substance and expressed by the "sieve-coefficient" C_L/C_P (C_L and C_P = concentration of dextran in lymph and plasma respectively) The transcapillary pressure difference was changed by altering the central venous pressure. In this way it was possible to change the transport rate of fluid and test molecules and gain new information about the functional ultrastructure of the blood-lymph barrier

Part III contains an analysis of the dextran sieving data from part II in terms of a two pore model and a model including vesicular transport. Estimates of pore and vesicular radii density ratios and in addition, the mean transcapillary pressure difference were calculated for "normal" conditions as well as after increased venous pressure. The models were tested not only for compatibility to data, but also for

adequacy (see below) this infers restrictions on the precision of the parameters of the model. The sieving data recorded under increased venous pressure, and the corresponding lymph flow data were found to be critical for the estimation of the various parameters of the models.

I. A theoretical model for analysis of macro-molecular sieving-data

The pore theory of capillary exchange, as introduced by Pappenheimer and his coworkers (1948, 1951 and 1953) is based on the concept of "equivalent pores" through which the molecules are transported by diffusion and filtration processes. The corresponding mathematical model for the "sieve ratio C_L/C_P includes the restriction to passage of molecules through cylindrical pores which are perpendicular to the capillary surface but is not derived from any other *explicit* assumptions about the structure of the capillaries. The derivation is, however based on the *implicit* assumption that the exchange conditions for fluid and macromolecules are the same throughout the whole capillary.

Winne (1965) derived more general equations for the sieve ratio C_L/C_P by starting from *a model capillary* of cylindrical shape. The pores were assumed to be isotropically distributed over the capillary area and the hydrostatic pressure was assumed to vary along the capillary length. The main advantage of Winne's model is its potential of treating the problem of reabsorption of fluid at the venous end of the capillary. However the model of Winne did not take into account *differences in permeability and surface area along the capillary* the importance of which has been pointed out by Wiederhielm (1968) in a very instructive analogue computer simulation of the capillary bed.

In this part I the model of Winne will be further developed to include these factors and the equations will be used for testing the influence of various realistic assumptions about the differences in porosity surface area and pressure conditions on the shape of the C_L/C_P-curve. This has not been done in previous studies.

A. Model concepts and assumptions

The complex structure of the capillary bed is schematically given in fig. 1a, showing an arterio-venular capillary along which the true capillaries ramify. The difference between hydrostatic and colloid osmotic pressure components determine the exchange of water in this system. In some capillary regions the hydrostatic and colloid osmotic pressure difference $\Delta\Pi$ is positive, which results in a net outward flow (= filtration) while in other regions there is an excess of colloid osmotic pressure over hydrostatic pressure resulting in an inward flow (= reabsorption). The various pressure parameters are given in fig. 1b. In the following model the actual distribution of capillaries is represented by *one* model capillary of length l, along which the hydrostatic pressure on the plasma side (P^*) is assumed to decrease linearly from the arterial to the venous end.

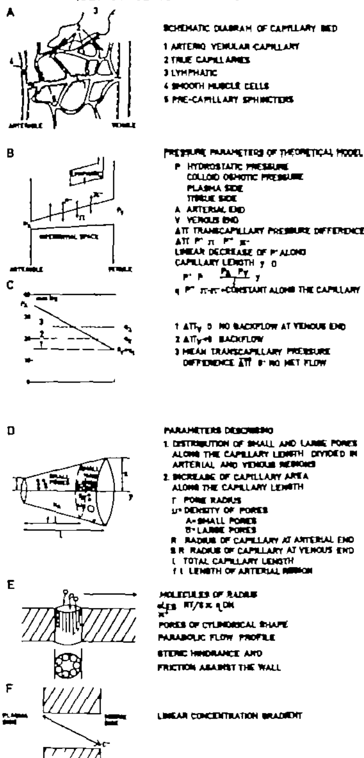


Fig. 1 Model concepts and assumptions made in the derivation of the general equations for the transport of macromolecules across the blood-lymph barrier

The interstitial hydrostatic pressure (P') and the colloid osmotic pressures (π and π ') are treated as constant along the whole capillary length. The condition for reabsorption on the venous side can then be stated $\Delta\Pi = P_V - P' - \pi + \pi < 0$ see case 2 in Fig 1 c. When $\Delta\Pi_V = 0$ no back flow occurs and when *the mean pressure difference* $\overline{\Delta\Pi} = 0.5\ (P_A + P_V) - P' - \pi + \pi$ is equal to zero, no *net* flow is possible.

In order to include *differences in porosity along the capillary* the model capillary was divided in two regions the arterial region of the length fxl ($0 \leqslant f \leqslant 1$) and the venous region, with different porosities due to different pore densities (ν) and sizes (r) of small (A) and large (B) pores (see fig 1 d) *The linear increase in surface area,* going from the arterial to the venous end of the capillaries (cf. Wiedeman, 1967) was handled by treating the model capillary as a truncated cone with the radius R at the arterial end and the radius SxR ($S \geqslant 1$) at the venous end (fig 1 d)

The pores are assumed to be of cylindrical form and penetrating the capillary wall perpendicular to the membrane (fig 1 e) The radius of such a pore was introduced by Paganelli and Solomon (1957) as the *equivalent pore radius* which was meant to be regarded as "an attempt to describe in operational terms a physical property of a complex biological membrane This equivalent pore concept has since then been found to be a self-consistent, and therefore useful, concept in the study of both artificial and biological (red cell) membranes (Solomon, 1969)

The dextran molecules are regarded as hydrated spheres, with an "effective" radius related to the diffusion coefficient D by Einstein-Stokes relation

$$a_{ES} = \frac{RT}{6\pi\ \eta DN} \qquad (1)$$

R = gas constant, T = absolute temperature, η = the viscosity of plasma, N = Avogadros number and π = 3 14159

The flow through the pores is governed by Poiseuille s law which has been found to be applicable for pore dimensions down to 10—15 Å in radius (see Solomon 1969) i.e. the flow is proportional to the pressure difference and the fourth power of the pore radius. The flow profile is parabolic, a circumstance that is difficult to realize in cylinders of the actual size, but is perhaps better grasped from a statistical point of view

The molecules are restricted in their passage through the pores firstly due to steric hindrance at the entrance of the pore and secondly due to friction against the walls of the pore The restriction factor is usually calculated according to the well-known semi-empirical formula (see e.g Renkin, 1954)

$$\beta = \{2(1-\alpha/r) - (1-\alpha/\ )\}\{1-2.104(\alpha/r)+2\ 09(\alpha/r) - 0.95(\alpha/r)\} \qquad (2)$$

where α is the radius of the molecule and r is the radius of the pore.

B Derivation of the equations of the model

The following derivation is an extension of the model given by Winne 1965

The transport of a water-soluble substance through a membrane of area F and with a pore density ν is given by the sum of the contributions from diffusion and filtration.

$$n = -D\beta\nu\pi^* r^2 \ dc/dx \ F + \beta\dot{V}c \tag{3}$$

where n flux of substance (mol/sec)
- D diffusion coefficient (cm /sec)
- β = restriction factor
- r = pore radius (cm)
- π^* = 3 14159
- ν = pore density (cm^{-2})
- c = concentration of substance (mol/ml)
- x = distance from aperture of the pore (cm)
- η = viscosity (atm sec)
- Δx = pore length (cm)

The net flux of water is closely approximated by

$$\dot{V} = (\pi^* r^4 \nu)/(8\eta\Delta x) \ F \ \Delta\Pi \tag{4}$$

where $\Delta\Pi$ is the hydrostatic and colloid osmotic pressure difference.

$$\Delta\Pi = P' - P'' - \tau + \pi \tag{5}$$

The net transport of water by diffusion is negligible see e.g Renkin, Pappenheimer 1957 The transport of dextran molecules of class (i) through the capillary of length l in fig 1 d is found by integration. The concentration is assumed to be constant along the capillary length both on the plasma (c') and tissue side (c'') The concentration gradient dc/dx along the pore is treated as linear

$$dc/dx = (c'' - c')/\Delta x \tag{6}$$

and the concentration in the filtration term is approximated by the mean value

$$c = (c' + c'')/2 \tag{7}$$

The elementary integration area dF is a function of distance y from the arterial end (y = 0)

$$dF = 2\pi^* R(y \frac{f-1}{l} + 1) \ dy \tag{8}$$

The diffusion and filtration "coefficients" K_{Di} K_{Fi} and K_{Fw} are also functions of y and by the assumption of two regions of different porosity the arterial region (*) of length f l and the venous region (**) of length $(1-f)$ l these coefficients can be written

$$K_{Di} = \begin{cases} K_{Di}^* = D_i\pi^*/\Delta x(\nu_A^* r_A^* \ \beta_{Ai}^* + \nu_B^* r_B^* \ \beta_{Bi}^*) & y \leq f\,l \quad (9\,a) \\ K_{Di}^{**} = D_i\pi^*/\Delta x(\nu_A^{*} \ r_A \ ^* \ \beta_{Ai}^{**} + \nu_B^{**} r_B \quad \beta_{Bi}^{**}) & y > f\,l \quad (9\,b) \end{cases}$$

$$K_{Fi} = \begin{cases} K_{Fi}^* = \pi^*/(8\eta \Delta x)(r_A^* r_A^{*\prime} \beta_{Ai}^* + r_B^* r_B^* \beta_{Bi}^*) & y \leqslant f\,l \quad (9\,c) \\ K_{Fi}^{**} = \pi^*/(8\eta \Delta x)(r_A^{**} r_A^{***} \beta_{Ai}^{**} + r_B \quad r_B^{***} \beta_{Bi}^* \ ) & y > f\,l \quad (9\,d) \end{cases}$$

$$K_{Fw} = \begin{cases} K_{Fw}^* = \pi^*/(8\eta \Delta x)(r_A^* r_A^{*\prime} \beta_{Aw}^* + r_{B*} r_B \quad \beta_{Bw}^*) & y \leqslant f\,l \quad (9\,e) \\ & w = \text{index for water} \\ K_{Fw}^* \quad = \pi^*/(8\eta \Delta x)(r_A \quad r_A^* \quad \beta_{Aw} \ ^* + r_B^{**} r_B^{**} \beta_{Bw}^{**}) & y > f\,l \quad (9\,f) \end{cases}$$

The net amount of molecules of class (i) transported per unit time across the capillary wall can then be written

$$n_i = \int_s \{(c_i - c_i) K_{Di} + (c_i + c_i)/2 \ K_{Fi} \ \Delta \Pi\} dF \tag{10}$$

where $\Delta \Pi = P_A - y(P_A - P_V)/l - q$ (11 a)

$$q = P' + \pi - \pi \quad = \text{constant} \tag{11 b}$$

After integration, eq (10) can be written

$$n_i = (c_i - c''_i) \ 2\pi^* \ Rl\{K_{Di} \ (f + \frac{s-1}{2} f^s) + K_{Di}^{**}(1 - f + \frac{s-1}{2}(1 - f^s)\} +$$

$$+ \frac{c'_i + c'_i}{2} \ 2\pi \ Rl(P_i \ K_{Fi} \ + P \ K_{Fi}^* \ ) \tag{12}$$

where

$$P \ = (P_A - q)(f + f^s \ \frac{s-1}{2}) - (P_A - P_V)(\frac{1}{2} f \ + \frac{s-1}{3} f^s) \tag{13 a}$$

$$P \ = (P_A - q)(1 + \frac{s-1}{2} - f - \frac{s-1}{2} f^s) -$$

$$- (P_A - P_V)(\frac{1}{2} + \frac{s-1}{3} - \frac{1}{2} f^s - \frac{s-1}{3} f^s) \tag{13 b}$$

The concentration c on the tissue side is given by $c_i = n_i/V_w$ (14)
where

$$\dot{V}_w \simeq 2\pi^* \ Rl \ (P \ K_{Fw}^* + P \ K_{Fw}^* \ ) \tag{15}$$

From eqs. (12)—(15) the lymph/plasma concentration ratio $\lambda_i = c_i/c_i$ can be solved

$$\lambda_i = \frac{DIFF_i + FILT_i/2}{FILT_w + DIFF_i - FILT_i/2} \tag{16}$$

$$DIFF_i = K_{Di} \ (f + \frac{s-1}{2} f^s) + K_{Di} \ ^*(1 - f + \frac{s-1}{2}(1 - f^s)) \tag{17 a}$$

$$FILT_i = K_{Fi}^* \ P + K_{Fi} \quad P \tag{17 b}$$

$$FILT_w = K_{Fw} \quad P + K_{Fw}^{**} \ P \tag{17 c}$$

The model contains a number of parameters specifying
pore radii r_A^* r_B^* r_A^{**} and r_B^{**}
pore density ratios ν_B^*/ν_A^* ν_A^{**}/ν_A^* and ν_B^{**}/ν_A^*
regional differencies in porosity f
difference in capillary area along the capillary length S
pressure conditions $P_A—P_V$ and $P_A—q$
It is to be noticed that the capillary length l, the capillary radius R and the thickness Δx of the capillary are eliminated in the expression for λ.

C. The influence of certain assumptions about differences in porosity surface area and pressure conditions along the capillary

The complexity of the model must be adjusted to the requirements of the experimental data and, at the same time, one has to minimize complexity by making simplifications in order to get working hypotheses. One of the main purposes of the model is to serve as a tool for looking into the consequences of one s assumptions. The influence of various approximations may be investigated and in this way the model focus attention on details that require further study

Various simplified models can be derived from the general expressions (11)—(17) e.g f = 1 S = 1 give a cylindrical capillary with isotropic distribution of small and large pores. The sieve ratio is given by

$$\lambda_i = \{0.5(r_A^4\beta_{Ai}+\nu_B/\nu_A r_B^4\beta_{Bi})+(D_i 8\eta/\overline{\Delta\Pi})(r_A^4\beta_{Ai}+\nu_B/\nu_A r_B\ \beta_{Bi})\}/$$
$$\{(r_A^4\beta_{Aw}+\nu_B/\nu_A r_B\ \beta_{Bw})—0.5(r_A^4\beta_{Ai}+\nu_B/\nu_A r_B\ \beta_{Bi})+$$
$$+(D_i 8\eta/\overline{\Delta\Pi})(r_A^4\beta_{Ai}+\nu_B/\nu_A r_B\ \beta_{Bi})\} \quad (18)$$

In this model there is only one pressure parameter $\overline{\Delta\Pi}$ the *mean* transcapillary hydrostatic and colloid osmotic pressure difference. This means that *net* filtration is calculated and reabsorption is not distinguished in this model. In the model by Winne (1965) reabsorption is included by using c as an approximation for concentration of outward filtration and c′ for reabsorption. In this way one more pressure parameter Π is introduced

$$\Pi = 0.5(P_A—\pi—P'+\pi')/(P_A—P_V) \quad (19)$$

and the sieve ratio is given by

$$\lambda_i = \{(\Pi_i/\overline{\Delta\Pi})(r_A\ \beta_{Ai}+\nu_B/\nu_A r_B\ \beta_{Bi})+(D_i 8\eta/\overline{\Delta\Pi})(r_A\ \beta_{Ai}+\nu_B/\nu_A r_B\ \beta_{Bi})\}/$$
$$\{(r_A\ \beta_{Aw}+\nu_B/\nu_A r_B\ \beta_{Bw})+(\Pi_i/\overline{\Delta\Pi}—1)(r_A^4\beta_{Ai}+\nu_B/\nu_A r_B\ \beta_{Bi})+$$
$$+(D_i 8\eta/\overline{\Delta\Pi})(r_A\ \beta_{Ai}+\nu_B/\nu_A r_B\ \beta_{Bi})\} \quad (20)$$

In the present investigation this model was used to test *the influence* of reabsorption, which occurs for $\Pi > \overline{\Delta\Pi}$ Figure 2 illustrates the influence of reabsorption on the sieve-ratio C_L/C_P The values for the arterial and the venous hydrostatic pressure were chosen in the range commonly accepted in the litterature. The values for the other parameters were chosen in the range calculated in part III For comparison

figs. 3 a—d show the influence of changes in the parameters r_A, r_B, ν_B/ν_A and $\overline{\Delta\Pi}$. As can be seen from these figures r_A and $\overline{\Delta\Pi}$ are mainly determined by the slope and position of the first part of the C_L/C_P curve (10—30 000) while r_B and ν_B/ν_A influence on the position of the second part of the curve. The variation in C_L/C_P due to various degree of reabsorption is equivalent to small changes in r_B and/or ν_B/ν_A. Thus the least-square estimates of the parameters r_B and ν_B/ν_A as obtained by using eq. (18) may be slightly *overestimated* if reabsorption is present.

The influence of various assumptions about *differences in porosity along the capillary* i.e. the parameter f and the paramters ν_B^*/ν_A^*, ν_A^{**}/ν_A^* and ν_B^{**}/ν_A^* was investigated by using eqs. (11)—(17). The results are illustrated in fig. 4. The model capillary has been divided in two parts (f): one penetrated by small pores ($\nu_B^*/\nu_A^* = 0$) and the other penetrated by additional large pores ($\nu_A^{**}/\nu_A^* \neq 0$, $\nu_B^{**}/\nu_A^* \neq 0$) and comparison is made with C_L/C_P values corresponding to an isotropic distribution of small and large pores all over the capillary surface. As can be seen from this figure (cp. figs. 3 b, c) the estimates of r_B and ν_B/ν_A are influenced by the specific assumptions about regional differences in porosity. In a least-square analysis using eq. (18) these parameters will be *underestimated*. The assumptions about pressure conditions are not critical for this problem.

The influence of various assumptions about *differences in surface area along the capillary* on the sieve coefficient was investigated in the same way by varying the radius of the capillary at the venous end ($1 \leqslant S \leqslant 5$) under various pressure conditions. An isotropic distribution of the pores was used. The results are shown in figs. 5 a, b. As can be seen from fig. 5 a the choice of S is not critical for the sieve ratio for small pressure gradients $d(\Delta\Pi)/dy$ but for larger gradients this parameter S performs a stronger influence (fig. 5 b) on the estimates of r_B and ν_B/ν_A. The assumption concerning reabsorption is thus critical for r_B and ν_B/ν_A in combination with assumptions about the variation of surface area along the capillary. In a least-square analysis using eq. (18) these parameters will be *overestimated*.

As can be seen from figs. 4 and 5 the effects of the simplifications mentioned above concerning differences in porosity, surface area and pressure conditions along the capillary counteract each other. The exact combined effect is difficult to predict, but from changes in the shape of the C_L/C_P-curve it is seen to influence on the estimates of r_B and ν_B/ν_A. The parameters r_A and $\overline{\Delta\Pi}$ are also influenced by the simplifications above when lymph flow data are considered.

The *concept of spherical molecules* may seem to be a rough approximation with great influence on the shape of the C_L/C_P-curve (fig. 6) and the conclusions about pore radii. It must however be emphasized that (1) the Einstein-Stoke's relation gives an "effective" radius of the molecule well suited for the description of the diffusion of the molecule, (2) the shape of the molecule must be discussed *in connection with* assumptions about the geometrical characteristics of the pores, i.e. the restriction factors. Fig. 6 is not fair in this respect. (3) the applicability of the concepts of "equivalent pores" and "effective radii" of molecules seems well justified in view of the ability of these concepts to provide self-consistent descriptions of artificial as

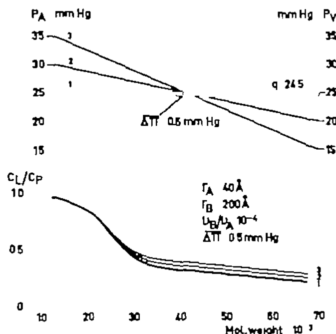


Fig. 2 The influence of various degree of reabsorption on the shape of the C_L/C_P curve.

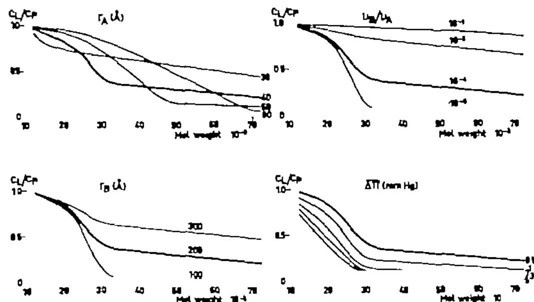
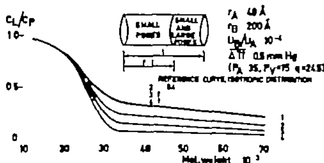


Fig. 3 Illustrates the influence of the model parameters r_A r_B, υ_B/υ_A and $\overline{\Delta\Pi}$ on the C_L/C_P curve. The parameters are varied one at a time with the following base setting $r_A = 40$ Å, $r_B = 200$ Å, $\upsilon_B/\upsilon_A = 10^{-4}$ and $\overline{\Delta\Pi} = 0.5$ mm Hg. The corresponding reference curve is indicated by a broad line in the figures.

Fig 4 illustrates the influence of the parameter f, i.e. various assumptions about regional differences in porosity The C_L/C_P curves are representative for the two different pressure conditions 1 and 3 indicated in fig 2



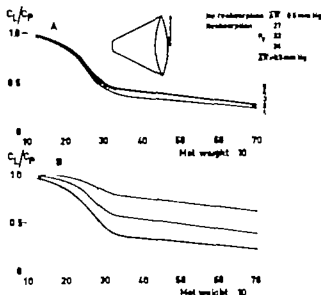


Fig 5 illustrates the influence of the parameter S, i.e. various assumptions bout variation of surface area along the capillary for a small ( ) and large pressure gradient (b)

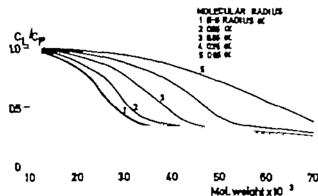
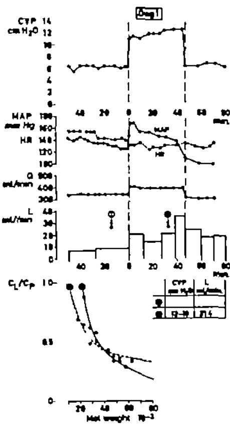
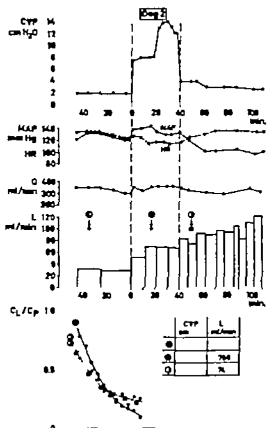
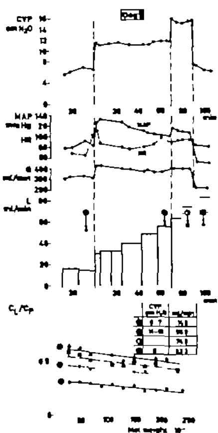
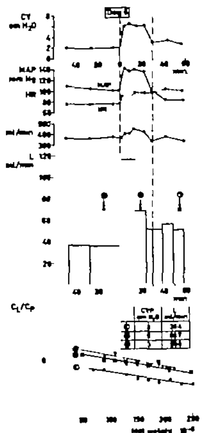


Fig 6 illustrates the influence of small changes in the radius of the molecule on the shape of the C_L/C_P curve. See comments in the text.


Dog 1
CVP cm H2O
MAP mm Hg
HR
Q ml/min
L ml/min
MAP
HR
min.
CL/CP
Mol weight 10^-3


Dog 2
CVP cm H2O
MAP mm Hg
HR
Q ml/min
L ml/min
MAP
HR
min.
CL/CP
Mol weight


Dog 3
CVP cm H2O
MAP mm Hg
HR
Q ml/min
L ml/min
MAP
HR
min
CL/CP
Mol weight


CV cm H2O
MAP mm Hg
HR
MAP
HR
ml/min
L ml/min
min
CL/CP
Mol weight

CVP was decreased again the various variables investigated also changed towards normal values. However the effect on the lymph flow varied (see discussion)

The P_{CO_2}, pH HCO^- and P_O did not change significantly during the experimental period

The total concentration of dextran in plasma and lymph slowly decreased with time. During periods of increased lymph flow the total concentration of dextran in heart lymph was only moderately influenced in contrast to the pronounced changes in the sieve coefficient (C_L/C_P) (Figs 7—10)

During normal venous pressure and normal lymph flow the sieve coefficient, C_L/C_P decreased with increasing molecular weight. During periods of increased lymph flow the C_L/C_P values were lower for molecular weights below 40 000 and higher for molecular weights above 40 000 (Figs 7—10)

Thus in the present experiments a significant influence of CVP upon the lymph flow and the 'sieve coefficient curve' has been demonstrated.

III. Computer analysis of data from dog heart-lymph experiments using theoretical models.

During normal venous pressure (CVP) and normal lymph flow the sieve coefficient C_L/C_P decreased with increasing molecular weight. During an increased CVP the C_L/C_P values, as compared to the normal C_L/C_P curve, decreased for molecular weights below 40 000 and increased for molecular weights above 40 000 (cf. Figs 7—10) These experimental findings and corresponding lymph flow data will be analysed by three different models for capillary exchange of macromolecules across the blood lymph barrier

A Models used in the analysis

The following mathematical models were used

1 The two-pore model described by eq (18) in part I

2 A model by Winne (1965) for transport by small pores and vesicles (cytopempsis)

The passive transport through large pores is replaced by the *active* transport by small vesicles of radius r_v. The corresponding equation for the sieve-ratio can be written

$$\lambda_i = \left\{(\Pi_i/\overline{\Delta\Pi})r_A\,\beta_{Ai}+(D_i 8\eta/\overline{\Delta\Pi})r_A\,\beta_{Ai}+4/3(\omega\ 8\eta\ \Delta x/\overline{\Delta\Pi}\ r_A)r^4x_i\right\}/$$
$$\left\{r_A\,\beta_{Aw}+(\Pi_i/\overline{\Delta\Pi}-1)r_A\,\beta_{Ai}+(D_i 8\eta/\overline{\Delta\Pi})r_A\,\beta_{Ai}+4/3(\omega\ 8\eta\ \Delta x/\overline{\Delta\Pi}\ r_A)r^4x_i\right\} \quad (21)$$

where ω is the number of vesicles which are transported in one direction per unit

Figs. 7—10 The experimental data from dog heart-lymph experiments. Dogs 1 2 5 and 6 are given in detail. The time course of central venous pressure (CVP) mean arterial pressure (MAP) heart rate (HR) cardiac output (Q) and lymph flow (L) is illustrated. The concentration ratio of dextran in lymph and plasma, C_L/C_P, is given in the normal state 0 and after increased central venous pressure ●.

time and unit area, Π_L is defined by eq (19) and ε_1 is the restriction factor for entrance in a vesicle. The *semi-empirical* expression for ε_1 is (Winne 1965)

$$\varepsilon_1 = \left\{2(1-a_1/r) - (1-a_1/r_s)^4\right\} \qquad (22)$$

3 A simple model by Renkin from which a permeability coefficient P = D $A_s/\Delta x$ can be evaluated from lymph flow (L) and C_L/C_P data

$$P = D_s\ A_s/\Delta x = L\ (C_L/C_P)/(1-C_L/C_P) \qquad (23)$$

where D is the free diffusion coefficient of solute and $A_s/\Delta x$ is the effective diffusion area per unit path length in the capillary membrane. This model, which is based on the assumption that dextran molecules are transported by diffusion only will be used as an independent check of the estimates of pore radii and density ratios as evaluated from the more detailed models used This model has also been used in an attempt to distinguish between transport through large pores versus vesicular transport (Renkin 1964)

B Computational methods

The various parameters used in the simplified models of the capillary wall, eqs. (18) and (21) will be treated as unknown parameters to be evaluated from the experimental data in a least-square analysis.

The parameters appear non linearly in the various models and can therefore, as is the case in the linear problem, not be calculated directly but only by an iterative procedure. The estimates of the parameters were calculated by a method due to Powell (1965) The function to be minimized was chosen to be the residual square sum

$$S(\theta) = \sum_{i=1} (\lambda_i^{calc} - \lambda_i^{exp})^2 \qquad (24)$$

where θ stands for the parameters. The final value of θ $\hat{\theta}$ is called a least square estimate It is to be noticed that this estimate is a maximum likelihood estimate, if the errors in the measured values are independent random variables from a Gaussian distribution with equal variance σ Because of the non-linearity one can however not be sure of the uniqueness of the converged estimates, it might well exist other and deeper local minima in the neighborhood.

Information about these matters is contained in the shape of the likelihood function (or the sum of squares function) in the p-dimensional parameter space (Box, 1960)

There is no sense to say that a model, out of many that gives the best fit is the physiologically most meaningful, because it is always possible to improve a fit by introducing more parameters. This, however results in an overspecification and an increased lack of precision in the estimated parameters, which may make them worthless.

The aim of the following computations has been to find an *adequate physical model* for the experimental data. The adequacy of a model means two things

1 the model is compatible with the data
2 the parameters of the model are well determined by the data.

Information on the compatibility is contained in the residuals $\lambda_i^{calc} - \lambda_i^{exp}$. Inspection of plots of these residuals reveals incompatibility in the form of systematic deviations from the assumed random pattern of the errors.

The precision of and the dependency between various parameters is most suitably obtained by studying the likelihood function (or the sum of squares function) and its shape in the parameter space. The region enclosed by the sum of squares contour S = S (θ)

$$S(\theta) \leqslant S(\hat{\theta}) \left(1 + \frac{p}{n-p} F(p\ n-p)\right) \tag{25}$$

gives an approximate 100 (1—α) % confidence region if the model is reasonably linear in θ within the region of interest (Beale 1960) F (p n—p) is the α significance level of the F distribution with p and n—p degrees of freedom. In a 2-dimensional case (p = 2) these contours are readily illustrated in a plane, but when the number of parameters increases the graphical illustration of the likelihood contours also becomes more tricky In the present investigation the multi dimensional sum of squares function (p = 7) was visualized by a number of 2-dimensional plots of suitable planes systematically chosen in the parameter space.

C. Results

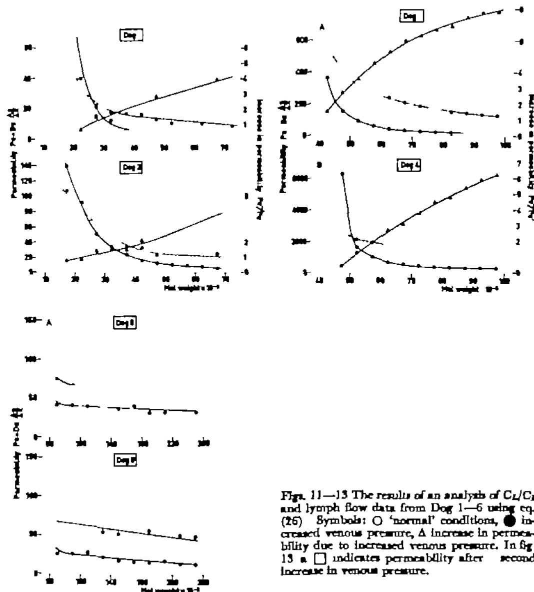
The C_L/C_P and the lymph flow data were first analysed by eq (23) based on the simple assumption that diffusion is the principal mechanism of transport of the dextran molecules through the capillary wall. As can be seen from Figs. 11—15 the effect of the increased venous pressure is, in all cases, an increased permeability P_s $(D_s \frac{A_s}{\Delta x})$ implying an increase in the effective pore area for the solute, A_s, and/or a decrease of the effective path length Δx

In terms of the two-pore model a large number of hypotheses of varying complexity can be formulated for the explanation of the experimental findings, eg

1 Are the changes in the C_L/C_P-data and the lymph flow *only* due to an increase in the capillary mean pressure difference $\overline{\Delta \Pi}$?

In order to test this hypothesis r_A (= r'_A and r''_A) r_B (= r'_B and r''_B) ν_B/ν_A (= ν'_B/ν'_A and ν''_B/ν''_A) $\overline{\Delta \Pi}'$ and $\overline{\Delta \Pi}''$ in eq (18) were treated as parameters and estimated by a least-square fit to the two experimental sets of data (lymph flow data included) The superscripts ' and '' now refer to the state before and after the increase in venous pressure, and not to the plasma and tissue side of the capillary As can be seen from Fig 14a it was not possible to get an acceptable match to the experimental points (dog 1)

2. Can the experimental data be accounted for by a change in the ratio ν_B/ν_A of



Figs. 11—15 The results of an analysis of C_L/C_P and lymph flow data from Dog 1—6 using eq. (26) Symbols: O 'normal' conditions, ● increased venous pressure, Δ increase in permeability due to increased venous pressure. In fig 15 a □ indicates permeability after second increase in venous pressure.

the density of small (ν_A) and large pores (ν_B)? The following parameters in the 2 pore model were estimated

r_A r_B, ν_B/ν_A ν_B/ν_A $\overline{\Delta \Pi}$ and $\overline{\Delta \Pi}$

Fig 14 b shows the incompatibility of this model as applied to the experimental data (dog 1)

3 Intuitively it seems plausible to assume that the dimensions of the pores are influenced by the increase in the venous pressure. This hypothesis was tested by using r_A r_A r_B r_B ν_B/ν_A, $\overline{\Delta \Pi}'$ and $\overline{\Delta \Pi}'$ as parameters in a concomitant least-square fit of eq (18) to the two sets of nerve data and the corresponding lymph flow data.

The increase in lymph flow was calculated as the ratio

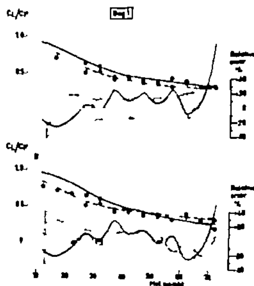


Fig 14 Comparison between experimental C_L/C_P-data for Dog 1 and corresponding calculated curves from an incompatible two-pore-model. Fig. 14 a shows the best fit according t alternative 1 in the text, while fig. 14 b illustrates the best fit according to alternative 2 Symbols: *experimental points;* 0 normal' conditions, ● increased venous pressure *calculated curves;* ——— normal' conditions, — — — increased venous pressure. The shaded area indicates the relative error in the experimental C_L/C_P-data.

$$\frac{(r_A \quad + \nu_B/\nu_A \; r_B \quad)\overline{\Delta \Pi'}}{(r_A \quad + \nu_B/\nu_A \; r_B \;)\overline{\Delta \Pi}} \tag{26 a}$$

under the assumption that the area for lymph collection is constant.

The compatibility of this model to the data from Dog 1 2 and 4 are illustrated in Figs. 15—17 The relative error curves fall inside the experimental error limits and the 2 pore model is therefore considered as compatible with the data. The corresponding point estimates of the parameters are given in Table I The change in permeability $D_B \frac{A_s}{\Delta x}$ due to changes in the radii and with the assumption that Δx is constant, was calculated as the ratio

$$\frac{\pi^* \; r_A{}'^2 \; \beta'_{A1} \; \nu_A + \pi^* \; r_B \quad \beta'_{B1} \; \nu_B}{\pi^* \; r_A \quad \beta'_{A1} \; \nu_A + \pi \; r_B \quad \beta'_{B1} \; \nu_B} \tag{26 b}$$

In Figs. 15—17 the estimated changes in permeability are compared with the independent estimates from the simple model of eq (23) The surprisingly good agreement confirms the reliability of the pore radii and relative number estimates. The importance of the lymph flow data as a further restriction on the model in the least square fit is illustrated by Fig 18, which shows a fit of eq (18) according to point 3 above without any concern about the increase in lymph flow As can be seen the compatibility to C_L/C_P data is satisfactory but the corresponding inrease in lymph flow and permeability is largely in error It is thus very important to test a model against all available relevant data and one must take every opportunity to place the model in jeopardy

Compatibility to data is however not a sufficient condition for the acceptance of a model. One must also investigate the confidence of the parameter estimates.

sure in Cat and Man with Special Reference to Shifts in Capillary Fluid Transfer Acta physiol scand. 1964 61 34—48.
Moore, D and H. Ruska, Fine structure of capillaries and small arteries. J biophys. biochem. Cytol. 1957 3 457—462
Paganelli, C. V and A. K. Solomon, The rate of exchange of tritiated water across the human red cell membrane. J gen. Physiol. 1957 41 259—277
Palade, G E., The endoplasmic reticulum. J biophys. biochem. Cytol. suppl. 1956 2. 85—98.
Pappenheimer, J R. and A. Soto-Rivera, Effective osmotic pressure of the plasma proteins and other quantities associated with the capillary circulation in the hindlimbs of cats and dogs. Amer J Physiol. 1948. 152 471—491
Pappenheimer, J R., E. M. Renkin and L. M. Borrero, Filtration, Diffusion and Molecular Sieving through Peripheral capillary Membranes. A contribution to the pore Theory of Capillary Permeability Amer J Physiol. 1951 167 13—46
Pappenheimer, J R., Passage of molecules through capillary walls. Physiol. Revs. 1953 33 387—423
Powell, M. J D., A method for minimizing a sum of squares of non-linear functions without calculating derivatives. Comput. J 1965 7 303—307
Renkin, E. M., Filtration, diffusion and molecular sieving through porous cellulose membranes. J gen. Phys. 1954 38. 225—243
Renkin E. M., Transport of Large Molecules across Capillary Walls. Physiologist, 1964 7 13—28.
Renkin, E. M. and J R. Pappenheimer, Wasserdurchlässigkeit und Permeabilität der Capillarwände. In Ergebnisse der Physiologie Biol. Chemie und Exp. Pharmak., O Krayer E. Lehnartz, A. v Muralt and H. H. Weber eds. Springer Verlag, Berlin Göttingen, Heidelberg, 1957
Solomon A. K. Characterization of biological membranes by equivalent pores. J Physiol. 1969 52. 335—364
Starling, E. H., On the absorption of fluids from the connective tissue spaces. J Physiol. 1896 19 312—326
Teorell, T., Kinetics of distribution of substances administered to the body I The extravascular modes of administration. II The intravascular modes of administration. Arch. int. Pharmacodyn. 1937 57 205
Wallenius, G Renal clearance of dextran as measure of glomerular permeability Acta Soc. Med. upsalien. Suppl. 39 1954 4 1—91
Wiederhielm C. A., Dynamics of Transcapillary Fluid Exchange. J gen. Physiol. 1968. 52. 29—63.
Wiederhielm, C. A., Analysis of small Vessel Function. In physical Bases of Circulatory Transport Regulation and Exchange. E. B Reeves and A. C. Guyton, eds. W B. Saunders Company Philadelphia, 1967 313—326
Wiedeman, M. P., Dimensions of blood vessels from distributing artery to collecting vein. Circulat. Res. 1963. 12. 375
Wiedeman, M. P Architecture of the terminal vascular bed. In Physical Bases of Circulatory Transport Regulation and Exchange. E. B. Reeves and A. C. Guyton, eds. W.B Saunders Company Philadelphia, 1967 307—312
Wiese, D Die Kapillarpermeabilität hochmolekularer Substanzen. Pflugers Arch. ges. Physiol. 1965 283 119—136.

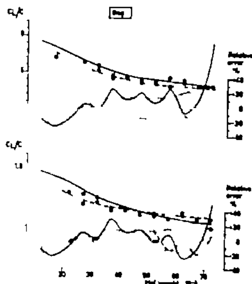


Fig 14 Comparison between experimental C_L/C_P-data for Dog 1 nd corresponding l- calated curves from an incompatible two-por -model. Fig. 14 shows the best fit' accordin to alternative 1 in the text while fig 14 l illustrates the 'best fit according t alternativ Symbols: *experimental points* 0 normal di tions, ● increased venous pressure *calculated curves* (——— normal' conditions, — — increased venous pressure The haded indicates the relativ error l the experime tal C_L/C_P-data.

$$\frac{(r_A \quad \tau \quad 1 \; \gamma_A \; r_B \quad) \overrightarrow{\Delta \Pi}}{(r_A \quad + \gamma_B \quad A \; r_B \quad \overrightarrow{1 \Pi'}} \tag{26 a}$$

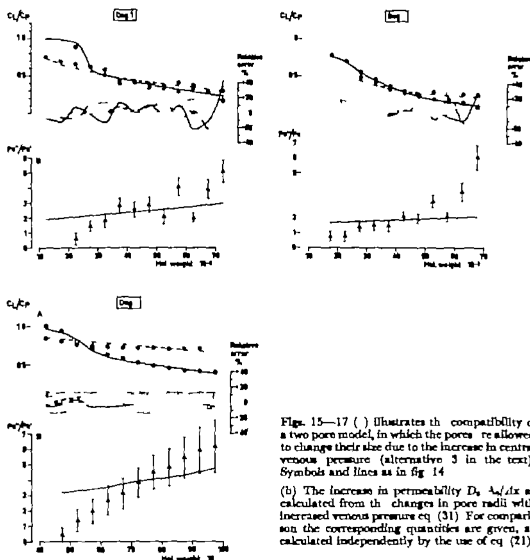
under the assumption that the area for lymph collection is constant.

The compatibility of this model to the data from Dog 1 2 and 4 are illustrated in Figs. 15—17 The relative error curves fall inside the experimental error limits and the 2 pore model is therefore considered as compatible with the data. The corresponding point estimates of the parameters are given in Table I The change in permeability $D_B \frac{A_s}{\Delta x}$ due to changes in the radii and with the assumption that Δx is constant, was calculated as the ratio

$$\frac{\tau \quad r_A \quad \beta'_{A1} \; \gamma_A + \pi^* \; r_B'' \quad \beta'_{B1} \; \gamma_B}{\tau^* \; r_A'' \; \beta'_{A1} \; \gamma_A + \pi^* r_B \quad \beta'_{B1} \; \gamma_B} \tag{26 b}$$

In Figs. 15—17 the estimated changes in permeability are compared with the independent estimates from the simple model of eq (23) The surprisingly good agreement confirms the reliability of the pore radii and relative number estimates. The importance of the lymph flow data as a further restriction on the model in the least square fit is illustrated by Fig 18 which shows a fit of eq (18) according to point 3 above without any concern about the increase in lymph flow As can be seen the compatibility to C_L/C_P data is satisfactory but the corresponding inrease in lymph flow and permeability is largely in error It is thus very important to test a model against all available relevant data and one must take every opportunity to place the model in jeopardy

Compatibility to data is however not a sufficient condition for the of a model. One must also investigate the confidence of the parameter esti



Figs. 15—17 ( ) illustrates th compatibility of a two pore model, in which the pores re allowed to change their size due to the increase in central venous pressure (alternative 3 in the text) Symbols and lines as in fig 14

(b) The increase in permeability $D_s\ A_s/\Delta x$ as calculated from th changes in pore radii with increased venous pressure eq (31) For comparison the corresponding quantities are given, as calculated independently by the use of eq (21)

TABLE I. Estimated parameter values from the simplified two-pore model, radii, and r_B, pore density ratio, ν_B/ν_A, nd mean transcapillary pressure difference, $\overline{\Delta \Pi}$ The estimates are given for normal nd levated central venous pressure (CVP) The corresponding lymph flow data (L) are also indicated (cf. Figs. 7 8)

	Dog 1		Dog 4		Dog 2	
r_A [Å]	38	(26)	53	(27)	57	(50)
r_B [Å]	141	(179)	145	(200)	175	(214)
ν_B/ν_A	4 10–[illegible]	(4 10–[illegible])	1 7 10–[illegible]	(1 7 10–[illegible])	1 6 10–[illegible]	(1 6 10–[illegible])
$\overline{\Delta \Pi}$ [cm H_2O]	6.10–[illegible]	(6.10–[illegible])	2.10–[illegible]	(3 7 10–[illegible])	3.6	(6.8)
CVP [cm H_2O]	6—7	(12—13)	7	(14)	2	(8)
L [ml/min]	9	(21 4)	260	(390)	31.9	(70 6)

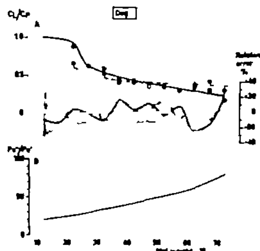


Fig 18 Illustrates the importance of lymph flow data in the least-square analysis. Th two-pore model, as conformed t the two sets of nerving data ( ) predicts an increase in lymph flow which is wrong by factor 100. This is reflected in the erroneous increase of the permeability coefficient (b)

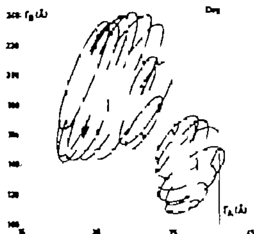
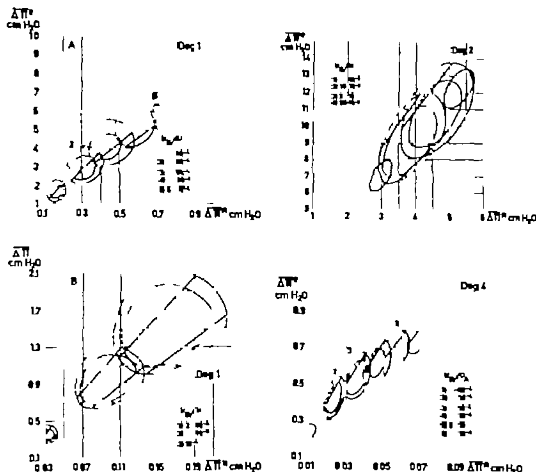


Fig 19 Dog 1: Projections of 99 % confidence regions on the r_A—r_B plane. The various indicated subregions correspond to various values of ν_B/ν $\overline{\Delta\Pi}$ and $\overline{\Delta\Pi}$ (see figs. 20—22) The point estimates of pore radii during normal and increased venous pressure are indicated +

The confidence limits of the parameters (Dog 1) are illustrated in Figs. 19 20 a, b as calculated from eq (25) The multi-dimensional parameter space was investigated systematically and the sum of squares function on the 99 % confidence level was illustrated by a number of 2-dimensional plots. Fig 19 shows the projection of the various sum of squares contours (Dog 1) on an r_A—r_B plane. The pore density ratio ν_B/ν_A and the pressure parameters $\overline{\Delta\Pi}$ and $\overline{\Delta\Pi'}$ were found to vary in the ranges indicated in Figs. 20 a, b These figures clearly demonstrate the high dependency and poor precision of the pressure parameters *in the case of Dog 1* The estimates of the pore radii are, however rather well determined. The non-linear constraints on the parameters, as imposed by the increase in lymph-flow are indicated in Figs. 20 a, b

A better estimation situation occurs for the cases of Dogs 2 and 4 as can be seen from Figs. 21 and 22. This is largely due to the smaller spread in the experimental C_L/C_P-



Figs. 20—22 99 and 95 % confidence regions for the pressure parameters $\overline{\Delta \Pi}$*(normal) and $\overline{\Delta \Pi}$ (elevated venous pressure) Constraints imposed by the lymph flow ratio ± 20 % are indicated.

points. A systematic investigation of the influence of errors, number and range of experimental points on the confidence limits is in preparation.

The model based on transport by small vesicles (cytopempsis) and small pores (eq 21) was conformed to the data of Dogs 1 2, 4 and found compatible to these data. This is illustrated in Figs. 23 24 and 25 The corresponding values of the parameters r_A r_A r_X r_X $\frac{\omega \Delta x}{\gamma}$ $\overline{\Delta \Pi}$ and $\overline{\Delta \Pi}$ are given in Table II As can be seen from this table and Figs. 26—28 the estimates of r_A r_A $\overline{\Delta \Pi}$ and $\overline{\Delta \Pi}$ are in close agreement with those obtained from the 2 pore model. The difference in estimation situation between Dog 1 and 2 is also the same as before, i.e. the estimates for Dog 2 are more precise. The estimates of small pore radii and radii of the vesicles in the case of Dog 1 are, however relatively well determined despite the lack of precision in the pressure difference. (See Fig 26 for Dog 1)

The small change in the radius of the vesicle, r_X due to an increase in the venous

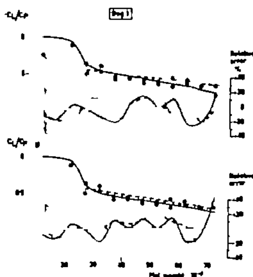
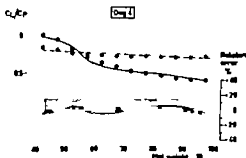


Fig. 23 shows the compatibility of the small-pore-vesicle model to the sieving data of dog 1

) the vesicle size is allowed to hange with pressure

b) the parameter w.d / is allowed t cha ge with pressure constant (cf. table II)





Figs. 24—25 show the compatibility of the small-pore-vesicle model to sieving data of dog 2 nd dog 4 (cf. table II)

pressure is not significant for Dog 1 Fig 23 b shows a compatible fit as calculated by treating r_Z and r_Z as one parameter r_Z, i.e. r_Z is not allowed to change with pressure increase. Instead the parameter $\frac{\omega\ \Delta x}{\gamma}$ was allowed to change with increased venous pressure. Table II gives the parameter values for this case.

Summary and discussion

In the present investigation of the blood-lymph barrier various theoretical models have been used to analyse macromolecular sieving-data as obtained from Starling dog heart lung preparations Various kinds of errors, originating from the experimental design, the model simplifications and the parameter analysis, were considered

The validity and the accuracy of the sieving data as a measure of the microvascular permeability seems well founded in regard of the performance of the dog heart lung

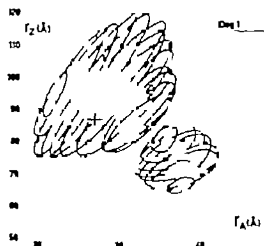
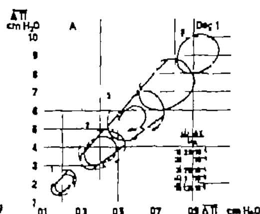


Fig 26 Dog 1: Projections of 99 % confidence regions on the r_A—r_Z plane. The various subregions indicated correspond to various values of ω Δ / $_A$ $\overline{\Delta\Pi}$ and $\overline{\Delta\Pi}$*(see figs. 27—28) The point estimates of pore and vesicle radius during 'normal nd increased venous pressure are indicated (+)



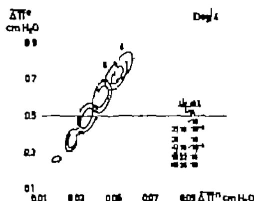


Fig 27—28. 99 % and 95 % confidence regions for the pressure parameters $\overline{\Delta\Pi}$ nd $\overline{\Delta\Pi}$ Constraints imposed by the lymph flow rati ±20 % are indicated.

TABLE II Estimated parameter values for simplified vesicular model; small pore radius, r_A, radius of vesicles, z, ratio of vesicular transport rate ω to small pore density and mean transcapillary pressure difference $\overline{\Delta\Pi}$ The estimates are given for "normal" nd elevated central venous pressure (CVP) The corresponding lymph flow data (L) are also indicated (cf. figs. 7 8 and table I) Dog 1a cf Fig 23a, Dog 1b cf Fig 23b.

	Dog 1		Dog 1b		Dog 4		Dog 2	
r_A [Å]	38	(27)	38	(27)	54	(32)	48	(46)
r_Z [Å]	74	(87)	81	(81)	91	(117)	77	(91)
$\frac{\omega\ \Delta x}{A}$	7.5 10⁻⁴	(7.5 10⁻⁴)	4.6 10⁻⁴	(1.2 10⁻⁴)	10⁻⁴	(10⁻⁴)	2.4 10⁻⁴	(2.4 10⁻⁴)
$\overline{\Delta\Pi}$ [cm H_2O]	6 10⁻⁴	(6 10⁻⁴)	7 10⁻	(6 10⁻)	2 10⁻⁴	(5 10⁻)	2.3	(4.8)
CVP [cm H_2O]	6—7	(12—13)	6—7	(12—13)	7	(14)	2	(8)
L [ml/min]	9	(21.4)	9	(21.4)	260	(590)	31.9	(70.6)

preparation and the high precision of the dextran distribution analysis. The dog heart lung preparation used in the present investigation is very suitable for studies of the blood-lymph barrier The tissue drained is homogenous and the area is fairly well-known (Allison and Sabiston jr 1957 Areskog *et al* 1964) The lymph flow is steady for several hours under normal conditions (Areskog *et al* 1964) and by altering venous pressure the lymph flow may be varied simultaneously (Arturson *et al.* 1968)

In the lymph sampling procedure a varying period of time will pass from the actual passage of a molecule through the vascular wall and to the moment at which it can be collected at the end of the lymph cannula. This sampling "delay time" can be approximately evaluated (Teorell, 1937) and has been estimated to be about ten minutes in the present heart-lymph experiments (cf Areskog *et al* 1964) This delay time will decrease with increased rate of lymph flow In view of the stability of the preparations, the small "delay time and the fact that the shape of the sieving curve is not markedly influenced by the falling plasma concentration of the test substance (Arfors *et al.* 1968 Arturson 1961 p 99) *steady state conditions are assumed* during the collection of plasma and lymph samples.

The dextran preparations used have been chosen in order to give optimal' informa tion about the capillary membrane, i e. to give sieve ratios in the complete range $0 \leq C_L/C_P \leq 1$ Furthermore, the test doses were given in small amounts since the concentration of dextran molecules in the lymph and plasma samples can be deter mined by the anthrone method with a very high precision This small dose minimizes the influence on the plasma volume.

The gel chromatography technique for distribution analysis of dextran has been tested and found to be superior to any conventional method of distribution analysis regarding the sensitivity reproducibility and resolving power (Granath & Laurent, 1967 Granath & Kvist, 1967 Arfors *et al* 1970) Within each dextran preparation used the molecular weight distribution for all molecular weight classes were deter mined, which provides a large number of measuring points on the sieve ratio curve.

Various models, based on the pore theory of capillary permeability were used in the analysis of the experimental data. The accuracy of the estimated model para meters is, of course, dependent on the various simplifications made in the model for mulation By using a general model (part I) the influence of assumptions concerning differences in porosity surface area and pressure conditions along the capillary was found to be of importance for the parameter estimates. Since the actual sieving and lymph flow data are not able to determine parameters on this level of resolution, a simplified model assuming a cylindrical capillary and an isotropic distribution of small and large pores, was used in the analysis. The estimated parameters must accordingly be regarded as equivalent parameters and the computational results must be interpreted in operational terms.

Explanations were sought for in terms of *adequate* models, i.e. besides the common condition of compatibility to data, an additional condition on the confidence of the parameter estimates was stressed. The adequacy of the models here used has been found to depend heavily on the sieving and lymph flow data obtained during

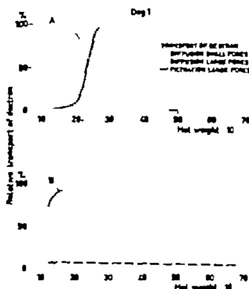
elevated central venous pressure. Fig 18 illustrates the importance of these data and the concept of adequacy In this figure the simplified two pore model has only been conformed to the two sets of sieving data. Despite the compatibility to these data (cf. fig 15 a, where also lymph-flow data were included in the fit) the estimated parameters implied an increase in lymph flow which was erroneous by a factor 100 This discrepancy to the lymph flow data is reflected in the erroneous increase in the permeability coefficient as can be seen in fig 18 b (cf. fig 15 b)

Under normal" conditions *the equivalent capillary membrane* can be characterized by the following estimates Small pore radius 35—60 Å, large pore radius 120—160 large to small pore density ratio around 10^{-4} and the mean transcapillary pressure difference less than 1 cm H_2O The *precision* of the parameter estimates varies from dog to dog beeing analyzed (see figs. 19—22) The *accuracy* of the parameter estimates is to some extent verified by the agreement between permeability coefficients derived from estimated pore radii and density ratios, and permeability coefficients estimated from the model of Renkin (cf. fig 15—17) The estimates of the parameters r_A, r_B and r_B/r_A are in the range previously reported in the literature (Pappenheimer *et al.* 1948 1951 1953 Grotte 1956 and Winne 1965) The mean transcapillary pressure difference calculated here ($\overline{\Delta \Pi} \leqslant 1$ cm of water) differs markedly from the estimate of 9 mm Hg which can be calculated from reported values for hydrostatic and colloid osmotic pressure components (see Wiederhielm 1968) This discrepancy may be due to the systematic errors introcued in the simplified model by assuming a constant permeability and a constant surface area along the capillary

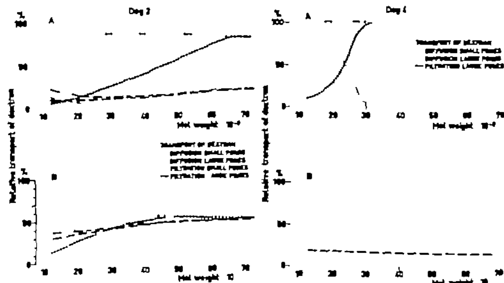
The *pressure perturbation data* give some interesting aspects of the function of the capillary bed. The (equivalent) effective filtration area for water A_w, as calculated from Poiseuille s law and parameter estimates from table I (Dog 1 and 4) is found to decrease to 1/2—1/3 of the normal value when the venous pressure is increased e.g Dog 1 $A_w \approx 27\,400$ cm normal, and $A_w = 13\,000$ cm during elevated CVP $\Delta x = 2000$ Å. This is caused by the pronounced decrease in small pore radius and a concomitant increase in the large pore radius. The changes in the estimated parameters seem to reflect adjustments of the vascular tone of the capillary bed, which have been found to take place, when a vascular bed is exposed to an increased transmural pressure By closure of a number of precapillary sphincter vessels, the size of the capillary surface is reduced" (Melander *et al* 1964 cf. Wiedeman 1967 and Thulesius, 1969) The observed increase in the lymph flow despite the reduced filtration area, is due to the increase in flow of fluid through the large pores. One per-cent of the flow was calculated to pass the large pores during normal conditions. The corresponding value during elevated CVP was found to be 9 (Dog 1) and 51 % (Dog 4) The mean transcapillary pressure difference $\overline{\Delta \Pi}$ was found to increase 5—10 of the increase in central venous pressure (Dog 1 and 4 figs. 20 22) Thus the large pores seem to function like "security valves through which fluid is transported to interstitial space by the elevation of central venous pressure. The concentration of colloids in the tissue space is then decreased, which results in a concomitant

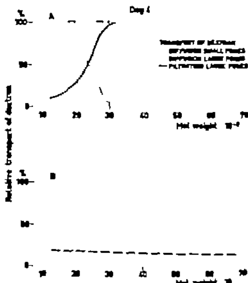
decrease in tissue colloid osmotic pressure (π'). The increase in the mean transcapillary pressure difference is then compensated for ($\overline{\Delta \Pi} = 0.5\,(P_A+P_V) - P' - \pi + \pi'$) and adjusted towards normal values. The conclusions which can be drawn from the calculated parameters thus support the hypotheses proposed by Wiederhielm (1968) in his analogue computer simulation of the transport mechanisms of the capillary bed.

The results of the analysis of Dog 2 differ markedly from the results concerning Dog 1 and Dog 4. For Dog 2 the effective filtration area A_w was found to be rather un-



Figs. 29—31 The relative transport of dextran molecules by diffusion and filtration through small and large pores. A) normal venous pressure B) elevated venous pressure.





sure in Cat and Man with Special Reference to Shifts in Capillary Fluid Transfer Acta physiol scand. 1964 61 34—48.

Moore, D and H. Ruska, Fine structure of capillaries and small arteries. J biophys. biochem. Cytol 1957 3 457—462

Paganelli, C. V and A. K. Solomon The rate of exchange of tritiated water across the human red cell membrane J gen. Physiol. 1957 41 259—277

Palade, G E., The endoplasmic reticulum. J biophys. biochem. Cytol. suppl. 1956. 2. 85—98.

Pappenheimer, J R. and A. Soto-Rivera, Effective osmotic pressure of the plasma proteins and other quantities associated with the capillary circulation in the hindlimbs of cats and dogs. Amer J Physiol. 1948. 152 471—491

Pappenheimer, J R., E. M. Renkin and L. M. Borrero, Filtration Diffusion and Molecular Sieving through Peripheral capillary Membranes. A contribution to the pore Theory of Capillary Permeability Amer J Physiol. 1951 167 13—46

Pappenheimer, J R., Passage of molecules through capillary walls. Physiol. Revs. 1953 33 387—423

Powell, M. J D A method for minimizing a sum of squares of non-linear functions without calculating derivatives. Comput. J 1965 7 303—307

Renkin, E. M., Filtration, diffusion and molecular sieving through porous cellulose membranes. J gen. Phys. 1954 38. 225—243

Renkin, E. M., Transport of Large Molecules cross Capillary Walls. Physiologist, 1964 7 13—28.

Renkin E. M and J R. Pappenheimer, Wasserdurchlässigkeit und Permeabilität der Capillarwände *In Ergebnisse der Physiologie, Biol. Chemie und Exp. Pharmak.,* O Krayer E. *Lehnartz,* A. Muralt nd H. H. Weber eds. Springer Verlag, Berlin Gottingen, Heidelberg 1957

Solomon A. K. Characterization of biological membranes by equivalent pores. J Physiol. 1969 52. 335—364

Starling E. H., On the bsorption of fluids from the connective tissue spaces. J Physiol. 1896. 19 312—326

Teorell, T Kinetics of distribution of substances dministered to the body I The extravascular modes of dministration. II The intravascular modes of administration. Arch. Int. Pharmacodyn. 1937 57 205

Wallenius, G Renal clearance of dextran as a measure of glomerular permeability Acta Soc. Med. upsalien. Suppl. 59 1954 4 1—91

Wiederhielm, C. A., Dynamics of Transcapillary Fluid Exchange. J gen. Physiol. 1968. 52 29—63

Wiederhielm C. A. Analysis of small Vessel Function. In physical Bases of Circulatory Transport Regulation and Exchange. E. B Reeves and A. C. Guyton, eds. W B. Saunders Company Philadelphia, 1967 313—326.

Wiedeman M. P Dimensions of blood vessels from distributing artery to collecting vein. Circulat. Res. 1963 12 375.

Wiedeman M. P Architecture of the terminal vascular bed. In Physical Bases of Circulatory Transport: Regulation and Exchange. E. B Reeves and A. C. Guyton, eds. W.B. Saunders Company Philadelphia, 1967 307—312.

W[illegible], D Die Kapillarpermeabilität hochmolekularer Substanzen. Pflugers Arch. ges. Physiol. 1965 283 119—136

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 375

CONVERGENCE ON INTERNEURONES IN THE RECIPROCAL Ia INHIBITORY PATHWAY TO MOTONEURONES

BY

HANS HULTBORN

GÖTEBORG 1972

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 375

From the Department of Physiology U[illegible]versity of Göteborg, Sweden

CONVERGENCE ON INTERNEURONES IN THE RECIPROCAL Ia INHIBITORY PATHWAY TO MOTONEURONES

BY
HANS HULTBORN

GÖTEBORG 1972

Contents

This thesis mainly constitutes a summary of the following articles

I Hultborn, H. and M. Udo Convergence of large muscle spindle (Ia) afferents at interneuronal level in the reciprocal Ia inhibitory pathway to motoneurones. Acta physiol. scand 1972. In press.

II. Fedina, L. and H Hultborn Facilitation from ipsilateral primary afferents of interneuronal transmission in the Ia inhibitory pathway to motoneurones. Acta physiol. scand. 1972. In press.

III Hultborn, H. and M. Udo Convergence in the reciprocal Ia inhibitory pathway of excitation from descending pathways and inhibition from motor axon collaterals. Acta physiol. scand. 1972. 84 95—108

IV Hultborn, H and M. Udo Recurrent depression from motor axon collaterals of supraspinal inhibition in motoneurones. Acta physiol. scand. 1972. In press.

V Hultborn, H., E. Jankowska and S Lindström, Relative contribution from different nerves to recurrent depression of Ia IPSPs in motoneurones. J Physiol. 1971 215 637—664

These papers are referred to by their Roman numerals in the text.

The following abbreviations are used postsynaptic potential PSP excitatory postsynaptic potential, EPSP inhibitory postsynaptic potential, IPSP recurrent inhibitory postsynaptic potential, RIPSP flexor reflex afferents, FRA.

General introduction

Lloyd (1943a, b, 1946a, b) established that electrical stimulation of large muscle afferents evokes not only monosynaptic excitation of motoneurones innervating the same and synergic muscles, but also short latency reciprocal inhibition of motoneurones to antagonists. He also demonstrated (Lloyd 1943c) that the excitatory two-neurone arc in fact forms the basis of the well known stretch reflex of Liddell and Sherrington (1924) Subsequently it has been established that the muscle afferents concerned in the stretch reflex and its reciprocal inhibition are those with annulospiral endings on muscle spindles — group Ia afferents (Granit 1950, Hunt 1952, Laporte and Bessou 1957 Eccles, Eccles and Lundberg 1957a, Lundberg and Winsbury 1960)

Lloyd (1941 1946a) originally proposed that the reciprocal inhibition from Ia afferents was exerted monosynaptically and he therefore coined the term "direct inhibition" Later studies proved, however that an interneurone must be interpolated in the Ia inhibitory pathway (Eccles, Fatt and Landgren 1956, R.M Eccles and Lundberg 1958a, Araki Eccles and Ito 1960, Jankowska and Roberts 1971 1972a, b) This interneurone was first merely considered as a commutator like device to transform the excitatory action of primary afferents to an inhibitory action (cf Eccles et al. 1956) but the question soon arose whether it also acts as a simple integrative centre (cf R.M Eccles and Lundberg 1958b) The latter idea has received strong support during the last decade from findings that volleys in cortico- rubro- and vestibulospinal tracts facilitate transmission in the Ia inhibitory pathway by excitation of the interposed interneurones (Lundberg and Voorhoeve 1962 Hongo, Jankowska and Lundberg 1969 Grillner Hongo and Lund 1966) Likewise it has recently been recognized that Ia inhibitory interneurones impinging on flexor motoneurones are facilitated in the crossed extensor reflex (Bruggencate, Burke, Lundberg and Udo 1969 Fedina and Hultborn 1972)

With the Golgi technique it has been established that axons of motoneurones often give off one or more collateral branches during their course through the ventral horn (Golgi 1903 Ramon y Cajal 1909 Prestige 1966 Szentagothai 1967 Scheibel and Scheibel 1971) Neurophysiologists did not hesitate to postulate functions from these anatomical findings and

it may be of interest in this connexion to recall that T Graham Brown (1914) suggested that reciprocal inhibition was mediated by these axon collaterals. It was not until 1941 however that Renshaw first described the now well known recurrent inhibition of motoneurones, which subsequently proved, beyond any reasonable doubt, to be produced by impulses travelling along motor axon collaterals (Eccles, Fatt and Koketsu 1954 cf Renshaw 1946) These impulses then activate specific interneurones, denoted Renshaw cells (Eccles et al. 1954) which in turn inhibit motoneurones.

Inhibition via motor axon collaterals was thus first described for α-motoneurones, but later investigations have demonstrated that γ-motoneurones are also inhibited by antidromic volleys in axons of α-motoneurones (Ellaway 1968 Brown Lawrence and Matthews 1968 Grillner 1969a, Ellaway 1971 Noth 1971) Recent studies have furthermore revealed that the Renshaw cells themselves receive inhibition following ventral root stimulation, thus demonstrating that the interneurones interposed in the recurrent pathway may inhibit each other (Ryall 1970 Ryall, Piercey and Polosa 1971)

As early as 1941 Renshaw had observed that antidromic impulses in motor axons not only caused inhibition of neighbouring motoneurones, but sometimes also facilitation. Subsequent studies have revealed that this recurrent facilitation in fact is caused by a release of motoneurones from a tonic inhibitory bombardment (Wilson 1959 Wilson and Burgess 1962) and it was postulated that this disinhibition is due to recurrent inhibition of tonically active inhibitory interneurones. It appeared possible that these interneurones which are inhibited during recurrent facilitation might be interposed in some inhibitory segmental reflex pathway to motoneurones. With this background Hultborn Jankowska and Lindström (1971a) investigated the effects of antidromic volleys in motor axons on reflex transmission from different types of primary afferents to motoneurones. It was found that conditioning ventral root stimulation effectively depressed transmission in the Ia inhibitory pathways to motoneurones It was disclosed that the depression is caused by postsynaptic inhibition of the interposed interneurones, which is evoked through recurrent motor axon collaterals and Renshaw cells (Hultborn et al. 1971a, b)

Aim of the present studies

Despite all results discussed above there are important gaps in our knowledge regarding the control of transmission in the Ia inhibitory pathway

In contrast to the detailed information concerning descending action (see Introduction) there is meagre knowledge concerning effects from various segmental afferents. The first part of the present studies therefore was aimed at analyzing the actions on the Ia inhibitory interneurones by volleys in ipsilateral segmental afferents.

The very finding of the extensive convergence from many neuronal systems on the Ia inhibitory interneurones (Introduction) raises the important question of whether all interneurones have a similar convergence or if there are functional subdivisions characterized by different patterns of convergence. In an attempt to elucidate this problem efforts were made to establish if supraspinal or segmental excitation and recurrent inhibition converge on the same Ia inhibitory interneurones.

The finding of inhibition of the Ia inhibitory interneurones by impulses in motor axon collaterals (Introduction) is of great interest from the view of regulation of transmission in the Ia inhibitory pathway as well as for the interpretation of effects from the recurrent collateral system. A crucial question to be solved before these aspects can be evaluated is whether the recurrent effects on transmission in afferent pathways to motoneurones are confined to the one mediating the reciprocal Ia inhibition. As a basis for a discussion of the functional significance of recurrent depression of reciprocal Ia inhibition, it is also necessary to determine the relative contribution from different efferent nerves to recurrent depression of Ia inhibition in various species of motoneurones.

The purpose of the present studies was thus

1 to extend the knowledge about the effects on Ia inhibitory interneurones from segmental afferents (I and II)

2. to show whether excitation from descending or segmental pathways on one hand and inhibition from recurrent motor axon collaterals on the other converge onto the same Ia inhibitory interneurones and thereby to tackle the problem of uniformity in the interneuronal pool mediating the reciprocal Ia inhibition (II and III)

3 to clarify whether recurrent inhibition of interneurones in segmental ipsilateral reflex pathways is restricted to those neurones mediating the reciprocal Ia inhibition (II)

4 to analyze the possibilities of using susceptibility to recurrent depression of segmental or descending IPSPs as an indicator that they are conveyed by the Ia inhibitory interneurones (II and IV)

5 to establish the relative contribution from different efferent nerves to the depression of transmission in Ia inhibitory pathways to different species of motoneurones (V)

6 to discuss the functional role of the convergence on the interneurones in the reciprocal Ia inhibitory pathway

Methodological considerations

Papers I—V are concerned with the convergence on the interneurones mediating reciprocal Ia inhibition to motoneurones supplying different hindlimb muscles in the cat. In this section will be considered only the principal experimental approaches employed in the analysis of interneuronal organization in reflex pathways. Detailed methodological descriptions are given in the respective papers.

A study of the synaptic actions mediated by polysynaptic pathways permits deductions about interneuronal organization in these pathways (cf Lundberg 1969a) All conclusions in the present series of papers (I—V) regarding the convergence on the interneurones in the Ia inhibitory pathway rest on studies of how Ia IPSPs evoked in motoneurones are influenced from other neuronal systems. A facilitation of a test Ia IPSP indicates an excitatory and a depression an inhibitory convergence Since the path way is disynaptic (see Introduction) these actions must be exerted on the interposed inhibitory interneurone. Any valid conclusion in this direction requires that the changes of the test IPSP are not caused by conductance or potential changes in the motoneurone recorded or to an interference at primary afferent level.

In the experiments described in papers I—IV the dorsal roots were kept intact while L5—S1 ventral roots were cut and mounted for stimulation and recording (left part of Fig. 1) The test Ia IPSPs were evoked by stimulation of Ia afferents in peripheral nerves from antagonists to the muscle supplied by the recorded motoneurone. Conditioning stimuli were given to skin, joint or other muscle nerves (I—II) or to supraspinal centres (III—IV) to test excitatory or inhibitory actions from their pathways onto the Ia inhibitory interneurones. When susceptibility to recurrent depression of various IPSPs was tested (II—IV) the conditioning antidromic stimuli were given to ventral roots in which volleys depressed transmission in the Ia inhibitory pathway without evoking any recurrent inhibition in the motoneurone recorded (cf Hultborn et. al. 1971a, papers III and V) Paper V deals with the relative contribution from efferent fibres in different nerves to the recurrent inhibition of Ia inhibitory interneurones. In that series of experiments the ventral roots were kept intact while the dorsal roots were cut and their central ends mounted for stimulation as shown

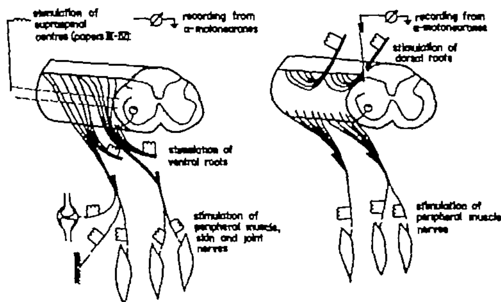


Fig. 1

Schematic representation of experimental arrangements. See text.

in the right part of Fig. 1 The test Ia IPSPs were evoked by stimulation of dorsal roots and the conditioning antidromic volleys by stimulation of motor fibres in different peripheral nerves.

The more direct approach to studying the interneuronal organization in reflex pathways, i.e. recording from the interneurones themselves is often hampered by the difficulty of identifying them as belonging to a defined pathway (see Lundberg 1969a). The present knowledge of the reciprocal Ia inhibitory interneurones obtained by the "indirect" approach (cf Eccles 1964 Lundberg 1970, Hultborn et al. 1971a) has permitted Hultborn et al. (1971b) to postulate that Ia coupled interneurones in the ventral horn, just dorsomedial to the motor nuclei, are mediating the disynaptic Ia inhibition. In fact, interneurones identified by their criteria were recently shown to produce monosynaptic IPSPs in motoneurones (Jankowska and Roberts 1971 1972a, b), thus finally proving that they mediate the reciprocal Ia inhibition to motoneurones. These workers, however were not able to demonstrate such an action from all Ia coupled interneurones fulfilling the criteria by Hultborn et al. (1971b) and therefore it is possible that some Ia coupled interneurones in the ventral horn are not interposed in the pathway of Ia reciprocal inhibition to motoneurones, but rather are involved in some other pathways.

Further studies on the Ia interneurones in the ventral horn will first of all show whether they are subject to all actions postulated in papers I—V Interneuronal recordings may also show whether excitatory actions from several supraspinal or segmental pathways converge onto the same Ia inhibitory interneurones, a question not easily solved by any other approach.

It is difficult to judge mixed excitatory and inhibitory effects from a neuronal pathway onto the Ia inhibitory interneurones by the indirect method with studies on test IPSP recorded in motoneurones (cf Discussion in paper II) Further interneuronal studies can also be expected to contribute new information regarding such questions. Reference will be made to such studies (Hultborn et al. 1971b, Hultborn and Santini 1972 and in progress)

When the terms facilitation and inhibition of reflex transmission are used they refer only to an excitatory and inhibitory convergence respectively in a pure anatomical sense. It is understood that findings from experiments of the present type cannot answer the question of how the convergence on the Ia inhibitory interneurones is actually used. Knowledge about excitatory and inhibitory convergence on interneurones in reflex pathways is, however necessary as a basis for tentative interpretations which subsequently can be tested by other experimental approaches.

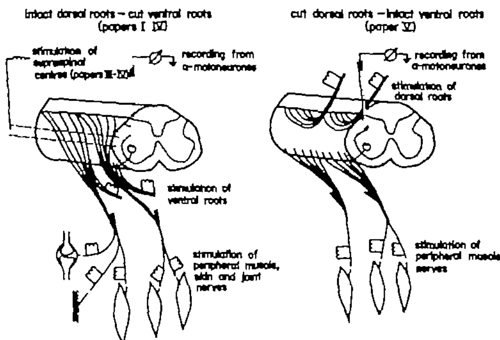


Fig 1

Schematic representation of experimental arrangements. See text.

in the right part of Fig 1 The test Ia IPSPs were evoked by stimulation of dorsal roots and the conditioning antidromic volleys by stimulation of motor fibres in different peripheral nerves.

The more direct approach to studying the interneuronal organization in reflex pathways, i.e. recording from the interneurones themselves, is often hampered by the difficulty of identifying them as belonging to defined pathway (see Lundberg 1969a) The present knowledge of the reciprocal I inhibitory interneurones obtained by the "indirect" approach (cf Eccles 1964 Lundberg 1970, Hultborn et al. 1971) has permitted Hultborn et al. (1971b) to postulate that Ia coupled interneurones in the ventral horn, just dorsomedial to the motor nuclei, are mediating the disynaptic Ia inhibition. In fact interneurones identified by their criteria were recently shown to produce monosynaptic IPSPs in motoneurones (Jankowska and Roberts 1971 1972a, b), thus finally proving that they mediate the reciprocal I inhibition to motoneurones. These workers, however were not able to demonstrate such an action from all Ia coupled interneurones fulfilling the criteria by Hultborn et al. (1971b) and therefore it is possible that some Ia coupled interneurones in the ventral horn are not interposed in the pathway of I reciprocal inhibition to motoneurones, but rather are invol ed in some other pathways.

Further studies on the Ia interneurones in the ventral horn will first of all show whether they are subject to all actions postulated in papers I—V Interneuronal recordings may also show whether excitatory actions from several supraspinal or segmental pathways converge onto the same Ia inhibitory interneurones, a question not readily solved by any other approach.

It is difficult to judge mixed excitatory and inhibitory effects from neuronal pathway onto the Ia inhibitory interneurones by the "indirect method with studies on test IPSP recorded in motoneurones (cf Discussion in paper II). Further interneuronal studies can also be expected to contribute new information regarding such questions. Reference will be made to such studies (Hultborn et al. 1971b, Hultborn and Santini 1972 and in progress)

ably will engage only fibres of spindle origin stronger stimuli will activate not only tendon organ fibres, but also many spindle afferents. Sumner (1961) reported similar findings for medial gastrocnemius, although the threshold difference for the lowest threshold spindle and tendon organ fibres was less than in the case of semitendinosus (cf also Granit 1970 p 104) With this background it is evidently very difficult to exclude ad ditional excitatory effects from Ib fibres on the Ia inhibitory interneurones. For this purpose a selective activation of Ib afferents would be desirable and the method of Coppin, Jack and McLennan (1970) seems to offer excellent possibilities.

3 *Ipsilateral high threshold (group II and III) muscle afferents and high threshold joint afferents* From the point of view of their reflex actions high threshold muscle afferents, high threshold joint afferents and cuta neous afferents are grouped together as flexor reflex afferents (FRA R.M Eccles and Lundberg 1959 Holmqvist Lundberg and Oscarsson 1960 Holmqvist and Lundberg 1961 Holmqvist 1961) The effects on the Ia inhibitory interneurones from cutaneous afferents will, however be described separately from those of the high threshold muscle and high threshold joint afferents since the cutaneous afferents in addition evoke effects through a neuronal pathway separate from the ipsilateral FRA pathway

In paper II it was shown that activation of the ipsilateral FRA pathway can facilitate transmission in the Ia inhibitory pathways to both flexor and extensor motoneurones in spinal cats under chloralose anesthesia. Facilita tion of Ia IPSPs was, however rather uncommon in the unanesthetized spinal state and always absent in decerebrate cats with a low pontine lesion (henceforth referred to as the low pontine preparation, Holmqvist and Lundberg 1961 see Methods in paper II) In the latter preparation, volleys in FRA regularly depressed the Ia IPSPs. Since this depression was not due to interference either at a motoneuronal or at a primary afferent level it was concluded that FRA volleys inhibit the Ia inhibitory interneurones in the low pontine preparation (paper II) The FRA inhibition of Ia inhibitory transmission in this preparation was seen in flexor as well as extensor motoneurones.

4 *Ipsilateral cutaneous afferents* Volleys in cutaneous afferents may facilitate transmission of Ia inhibition both to flexor and extensor motoneurones, although less frequently in quadriceps motoneurones than in other species of motoneurones tested (paper II) This facilitation could be evoked by a stimulus strength of about 1 15—1.2 × threshold for the lowest

threshold fibres in the cutaneous nerves and was then seen to grow with stimulus intensities up to 2—3×threshold. When the stimulus strength was raised above this value it was usually not possible to demonstrate any increasing facilitation. This does not exclude the possibility that higher threshold fibres contribute to the excitation of the Ia inhibitory interneurones — the failure may depend on a limited subliminal fringe leading to a more pronounced occlusion with increasing excitatory drive (also a consequence of excitatory convergence) (cf paper II)

The time course of the facilitation of Ia IPSPs following a cutaneous conditioning volley (Fig. 9 paper II) as well as direct interneuronal recording (Hultborn and Santini, to be published) suggests that the minimum excitatory linkage from cutaneous afferents to Ia inhibitory interneurones is not more than trisynaptic. The facilitatory action on Ia inhibitory transmission from cutaneous afferents could be demonstrated not only in spinal, chloralose anesthetized, but also in unanesthetized cats and in decerebrate (with or without a low pontine lesion) preparations. In the low pontine preparation there was no corresponding facilitation by volleys in high threshold muscle and joint afferents, which instead evoked a depression of transmission. It was therefore postulated (paper II) that the excitation of Ia inhibitory interneurones from low threshold cutaneous afferents is partly mediated by a separate reflex pathway in which transmission is not obliterated in the decerebrate state. The present results thus conform to earlier evidence (Hagbarth 1952 Engberg 1964 Hongo et al. 1969 Baldissera, Bruggencate and Lundberg 1971) showing that low threshold cutaneous afferents have separate reflex pathways to motoneurones in addition to the FRA pathway (R.M. Eccles and Lundberg 1959)

5 *Contralateral flexor reflex afferents* Bruggencate et al. (1969) reported that volleys in contralateral cutaneous and high threshold muscle afferents can evoke facilitation of transmission in the Ia inhibitory pathway to flexor motoneurones. It has now been shown (Fedina and Hultborn 1972) that while this facilitation of Ia IPSPs in flexor motoneurones from the contralateral FRA (cutaneous, high threshold muscle and joint afferents) is readily found in spinal chloralose anesthetized cats, it is much more difficult to demonstrate in the unanesthetized state and never observed in low pontine cats (with or without chloralose) There is a discrepancy between the results obtained with the indirect method (recording of Ia IPSPs in motoneurones) and the direct method (recording from the interneurones themselves) regarding effects from contralateral FRA on the Ia inhibitory transmission to extensor motoneurones. With the former method

there was scant evidence for any facilitation of Ia inhibitory transmission (Fedina and Hultborn 1972 Bruggencate, Lundberg and Udo to be published) while the latter approach revealed that interneurones activated from flexors (and thus presumably inhibiting extensor motoneurones) often receive excitation from the contralateral FRA (Hultborn et al. 1971b, Hultborn and Santini, to be published)

As for the ipsilateral FRA (paper II) it was shown that volleys in contra lateral FRA in the low pontine preparation depressed Ia transmission in motoneurones to flexors as well as extensors (Fedina and Hultborn 1972) These findings apply also to volleys in contralateral cutaneous afferents and there is thus no need to postulate any separate cutaneous reflex pathway having excitatory connexions with the Ia inhibitory interneurones as in case of the ipsilateral cutaneous afferents (cf above)

B. Is the interneuronal pool which mediates reciprocal Ia inhibition functionally homogeneous?

Numerous observations show that interneurones of reflex pathways to α motoneurones receive extensive convergence from other neuronal pathways than the primary afferents by which they are designated (cf e.g Lundberg 1969a) Disynaptic reciprocal Ia inhibition is thus facilitated from several descending and segmental pathways due to an excitatory convergence on the interposed interneurones (Introduction and section A in Results). A depression due to inhibition at an interneuronal level is likewise evoked from several neuronal systems, although most efficiently by volleys in ventral roots (Introduction and section A in Results) The pre sence of this extensive excitatory and inhibitory convergence raises the question of whether the whole interneuronal pool is functionally homogeneous — i.e. do all interneurones receive a similar convergence — or are there functional subdivisions characterized by differences in interneuronal convergence. In order to tackle this problem the method of using facilita tion and inhibition of PSPs as indicators of an excitatory and inhibitory convergence onto interneurones respectively was modified to allow judgements of convergence on interneurones from more than two systems (paper III) If a test PSP was evoked by spatial facilitation between two excita tory convergent systems a depression of this test from an inhibitory system allows the conclusion that all the three systems involved converge onto the same interneurones. In paper III it was established that excitation from either corticospinal, rubrospinal or vestibulospinal pathways on one hand,

and recurrent inhibition from motor axon collaterals on the other do converge onto the same Ia inhibitory interneurones. Similarly it could be demonstrated that recurrent inhibition and excitation either from the separate cutaneous pathway or from ipsi as well as contralateral FRA pathway converge onto the same Ia inhibitory interneurones (paper II Fedina and Hultborn 1972) These results prove the existence of Ia inhibitory interneurones which receive both excitation from a supraspinal or segmental pathway and recurrent inhibition from motor axon collaterals. Accordingly these excitatory and inhibitory systems can mutually interact in the control of transmission in the Ia inhibitory pathway However the employed technique does not allow us to exclude the existence of other groups of Ia inhibitory interneurones receiving either excitation from descending pathways or recurrent inhibition but not both Neither does it allow an investigation of whether several of these excitatory systems converge onto the same interneurones.

Regarding the first of these limitations, Hultborn et al. (1971b) discussed the possibility of two parallel Ia inhibitory pathways to motoneurones. Their finding that submaximal Ia IPSPs may be completely abolished by preceding antidromic ventral root volleys (Hultborn et al. 1971a) demonstrated that all interneurones responsible for these submaximal IPSPs did receive recurrent inhibition. It might be argued that there are two pools of interneurones one with a strong linkage, fired by submaximal Ia volleys and susceptible to recurrent inhibition, and another requiring more summation and lacking recurrent inhibition The sub-total depression of maximal Ia IPSPs is, however sufficiently explained by the observation that recurrent inhibition does not prevent maximal Ia volleys from activating most Ia inhibitory interneurones in which antidromic volleys obliterated spikes evoked by submaximal Ia volleys (Hultborn et al. 1971b Hultborn and Santini to be published) To sum up, hitherto there is no positive evidence for the existence of Ia inhibitory interneurones lacking inhibition from motor axon collaterals.

The presence or lack of excitatory convergence from several supraspinal or segmental systems on the Ia inhibitory interneurones cannot be gauged from work on Ia IPSPs in motoneurones (cf Methodological considerations) but here interneuronal recordings have contributed. A convergence of excitation from both the rubrospinal tract and pyramidal tract was proved common in interneurones receiving Ia excitation and recurrent inhibition (Hultborn and Santini 1972) Interneurones monosynaptically excited from the knee extensor quadriceps (and thus presumably inhibiting the knee flexor posterior biceps-semitendinosus) were usually also mono-

synaptically excited from the ipsilateral ventral quadrant in which the vestibulospinal tract descends (cf Grillner et al. 1966) Interneurones with a convergence of excitation from several supraspinal systems often received excitation also from the ipsilateral and/or contralateral FRA (Hultborn et al. 1971b Hultborn and Santini to be published) In summary there is a bulk of evidence demonstrating that excitatory as well as inhibitory actions from various neuronal systems which are known to affect Ia inhibitory transmission, in fact converge onto the same Ia inhibitory interneurones. *Hitherto there are no findings suggesting a functional subdivision within the interneuronal pool mediating Ia inhibition to a given motor nucleus* It cannot be excluded that further investigations may reveal a differentiation

C. Do interneurones in pathways to motoneurones other than those mediating the reciprocal Ia inhibition receive recurrent inhibition?

1 Effects on transmission from primary afferents The most striking effect of antidromic volleys in motor axons on reflex transmission to motoneuerones was the efficient depression of Ia IPSPs in all species of motoneurones tested (Hultborn et al. 1971a) It was, however noticed that in addition some IPSPs evoked from the ipsilateral FRA occasionally were depressed by volleys in ventral roots. IPSPs from Ib afferents and EPSPs from any afferent system were never affected. This finding of recurrent depression of FRA IPSPs may appear contradictory to the idea of a selective recurrent inhibition of the interneurones mediating the reciprocal Ia inhibition. It is therefore of interest to recall that FRA stimulation often can facilitate Ia IPSPs through spatial summation of the Ia inhibitory interneurones (see section A) Direct recording from interneurones supposed to mediate reciprocal Ia inhibition has in fact shown that volleys in the ipsilateral as well as contralateral FRA may cause a vigorous firing (Hultborn et al. 1971b, Hultborn and Santini, to be published) It was consequently suggested that the recurrent depression of FRA IPSPs was due to their partial transmission via the Ia inhibitory interneurones (Hultborn et al. 1971a) In order to give experimental support to this notion the recurrent depression of IPSPs from different primary afferents was correlated with the ability of volleys in the same afferents to facilitate transmission in the Ia inhibitory pathway (paper II, Fedina and Hultborn 1972) A strong positive correlation was established between susceptibility to recurrent depression of IPSPs from various ipsilateral (paper II) as well as contra

lateral afferents (Fedina and Hultborn 1972) and ability by volleys in these afferents to facilitate transmission in the Ia inhibitory pathway This means that an IPSP from a given group of afferents is depressed by ventral root stimulation only (with few exceptions) when a volley in these afferents causes facilitation of a test Ia IPSP This would support the idea that the recurrent inhibition of interneurones in segmental afferent pathways to motoneurones is restricted to those mediating the reciprocal Ia inhibition.

The recent findings of a mutual inhibition between Renshaw cells were reviewed in the Introduction. Since it has been postulated that ipsilateral segmental afferents may fire Renshaw cells "orthodromically" (i.e. not secondary to firing of motoneurones Eccles et al. 1954 Frank and Fuortes 1956, Curtis, Phillis and Watkins 1961) it would seem possible that they can transmit inhibition to motoneurones from primary afferents which thus would be susceptible to recurrent depression The question of whether the afferent facilitation of Renshaw cells shall be regarded as part of an "afferent pathway" to motoneurones or be interpreted as an afferent control of the recurrent pathway from motor axon collaterals is certainly more than a semantic problem, and one which cannot be solved by experiments designed to demonstrate an excitatory convergence.

2 *Effects on inhibitory transmission from supraspinal centres* It has been suggested that descending IPSPs in motoneurones are secondary to excitation of interneurones of segmental reflex pathways, but it cannot be excluded that they partly are conveyed also by "private" interneurones (i.e. interneurones activated by a supraspinal system, but unaffected by segmental reflex pathways for references see Discussion in paper IV) The question is now whether any such "private" interneurones (if at all existing) receive recurrent inhibition. When stimulation of supraspinal centres (cortico-rubro- and vestibulospinal pathways and a presumed reticulospinal pathway descending in the medial longitudinal fascicle) evoked IPSPs in motoneurones which were susceptible to recurrent depression these same stimuli invariably facilitated Ia IPSPs (see papers IV and III) Accordingly there are no observations which suggest recurrent inhibition of interneurones mediating descending IPSPs in motoneurones other than those interposed in the Ia inhibitory pathway (paper IV)

The results discussed in this section indicate that *there is no recurrent inhibition of interneurones in pathways to motoneurones other than those transmitting the reciprocal Ia inhibition and the recurrent inhibition*

D Does recurrent depression of IPSPs indicate that they are mediated by Ia inhibitory interneurones?

Since Renshaw cells also receive recurrent inhibition (Ryall 1970 Ryall et al. 1971) it is necessary to consider the possibility that segmental (paper II) or supraspinal (paper IV) IPSPs in motoneurones are mediated by these interneurones. A transmission via Renshaw cells can occur either by their orthodromic excitation or secondary to firing of motoneurones. In order to exclude the latter possibility it is important to control that the afferent or descending volleys used to evoke the test IPSP in the motoneurone does not elicit any discharge in ventral roots. The possibility that segmental afferent volleys or descending volleys may fire Renshaw cells orthodromically remains, but this is unlikely to contribute significantly to the IPSP recorded in the motoneurone at least in the case of the segmental and supraspinal pathways studied in paper II and IV (see the Discussions in the respective papers) If a small part of the IPSPs nevertheless was conveyed by Renshaw cells it is unlikely that even that part would be effectively depressed by conditioning ventral root volleys since the inhibitory interaction between Renshaw cells seems to be very weak (Hultborn, Jankowska, Lindström and Roberts 1971 see their Fig 6) These arguments strongly indicate that recurrent depression of IPSPs (which are not secondary to firing of motoneurones) reflects at least a partial mediation of these IPSPs by Ia inhibitory interneurones.

Does the lack of recurrent depression of an IPSP then exclude that it is mediated by the Ia inhibitory interneurones? An answer in the affirmative would require that *all* the interneurones mediating reciprocal Ia inhibition are susceptible to recurrent inhibition This has not been proved but there is, on the other hand, no positive evidence for the existence of Ia inhibitory interneurones lacking recurrent inhibition (see above in section B) Provided that (i) the conditioning ventral root volleys effectively depress Ia IPSPs in the motoneurone tested and that (ii) the segmental or supraspinal stimuli (used to evoke the IPSP to be tested) are graded to exclude that a failure to show a recurrent depression depends on a too strong excitatory drive onto the Ia inhibitory interneurones (cf Fig 1 in Hultborn et al 1971a) it is reasonable to conclude that a lack of recurrent depression virtually excludes that any significant part of such an IPSP is transmitted via the Ia inhibitory interneurones.

To summarize, *recurrent depression of IPSPs in motoneurones seems to indicate that — and also roughly to which extent — they are mediated by the Ia inhibitory interneurones*

E. Relative contribution from different nerves to recurrent depression of Ia IPSPs in motoneurones (paper V)

As a base for further attempts to analyze the functional significance of the recurrent control of the transmission in the Ia inhibitory pathway it seemed important to learn which motoneurone axon collaterals evoke depression of Ia IPSPs in different species of motoneurones. In order to obtain conditioning effects only from efferents when stimulating the peripheral nerves the dorsal roots were cut and the test Ia IPSP evoked by stimulation of the proximal ends of the dorsal roots (right part of Fig 1) By selection of dorsal roots used for stimulation and by careful grading of stimulus strengths it was possible to evoke pure or almost pure Ia IPSPs in many motoneurones (for criteria see Methods in paper V)

It was invariably found that the strongest depression of Ia IPSPs was evoked from motor fibres to muscles whose Ia afferents produce the IPSPs (Tables 1 2 and 4 in paper V) This means that the Ia IPSP e.g. from the knee extensor quadriceps muscle in a knee flexor posterior biceps-semitendinosus motoneurone is most effectively depressed by antidromic stimulation of motor fibres to the knee extensor (see the summarizing Fig. 8 in paper V) Although the maximal recurrent depression of IPSPs thus is always evoked from the nerves whose afferents give Ia inhibition recurrent effects are evoked also from some other nerves.

When the contribution from different nerves to recurrent inhibition was compared, a strong parallelism was revealed for motoneurones and Ia inhibitory interneurones receiving the same Ia excitatory input (Table 6, paper V) It was therefore concluded that the origin of recurrent inhibition of motoneurones and inhibitory interneurones supplied by the same Ia afferents is identical. With this background it was also suggested that the same Renshaw cells inhibit both motoneurones and Ia inhibitory interneurones.

Although recurrent inhibition of γ-motoneurones now has been established (see Introduction) there is still no extensive study on its origin in γ-motoneurones to different muscles. The results by Ellaway (1971) demonstrate, however that the strongest effects are exerted from the nerves to the muscle supplied by the γ-motoneurone or its synergists and it seems therefore likely that the recurrent effects in fact are exerted in parallel onto α- and γ-motoneurones supplying the same muscle. To summarize, *the prevailing pattern of recurrent effects indicates that activity in one motor nucleus will cause recurrent inhibition of α- and γ-motoneurones within that motor nucleus and other nuclei innervating synergists and also of Ia*

inhibitory interneurones impinging on motoneurones to antagonists as drawn in Fig 2A

Despite the striking similarities of recurrent inhibition in α-motoneurones nd I inhibitory interneurones, some differences have emerged from a recent in estigation by Burke, Fedina and Lundberg (1971). During controlled Cl^--injection in motoneurones they found that group Ia IPSPs reversed to a depolarizing potential with less amounts of Cl^- than was necessary to reverse the RIPSP completely This and other evidence led them to postulate that the synaptic terminals from Renshaw cells are located largely on the proximal dendrites of motoneurones, while the terminations of i terneurones generating the group Ia IPSPs (nd also those evoking IPSPs from several other peripheral afferent systems) appear to be closer to or on the cell somata. During incidental observ tions in Ia inhibitory interneurones (Burke et al. 1971) it was not possible to demonstrate a differential reversal between RIPSPs and other IPSPs. Furthermore the conductance changes underlying RIPSPs in these interneurones were larger and much more effecti in depressing Ia EPSPs than was the case in motoneurones.

General discussion

Functional aspects of the convergence onto the Ia inhibitory interneurones

A Reciprocal inhibition in α γ-linked movements

Liddell and Sherrington (1924 1925) demonstrated that a stretch of a muscle results in a prompt contraction which tends to restore it to its former length. Their concept of the stretch reflex was thus that of a negative feed back mechanism counteracting gravity The recognition of the fusi motor control of this feed back mechanism (Leksell 1945 Hunt and Kuffler 1951a,b) led Merton (1953) to present his follow up length servo hypothesis This hypothesis implies that the length to which a muscle is supposed to contract is set by the γ-activated intrafusal muscle fibres. The extrafusal muscle fibres were then assumed to be forced by the stretch reflex to contract — "to follow up — to the same "functional length" as the spindle.

Subsequent work (summarized by Granit 1955 1968 and 1970) has shown that numerous neuronal pathways evoked parallel effects on α- and γ-motoneurones which led Granit (1955) to postulate that movements often depend on a co-activation of α and γ-motoneurones According to this hypothesis a neuronal system with α-γ-linked" actions will exert synaptic depolarization of α motoneurones via two routes the "direct' α route and the "indirect γ-route (Fig 2B) This implies that the discharge of many α-motoneurones will depend on spatial facilitation from the "direct" α- and the "indirect" γ-routes. For a number of naturally occurring movements it has been possible to demonstrate a co-activation of α and γ-motoneurones so balanced that the afferent discharge from spindles actually increases during muscle shortening in quiet breathing (Eklund, Euler and Rutkowski 1964 Sears 1964) in stepping movements (Severin, Orlovskii and Shik 1967) in jaw movements (Taylor and Davey 1968) and finally in voluntary movements in man (Hagbarth and Vallbo 1968 1969 Vallbo 1971) This means that the tendency for unloading of muscle spindles during extrafusal shortening is well compensated by intrafusal contraction which will allow the stretch reflex to operate even during shortening If the shortening of a contracting muscle is opposed, an increased discharge in the Ia afferents

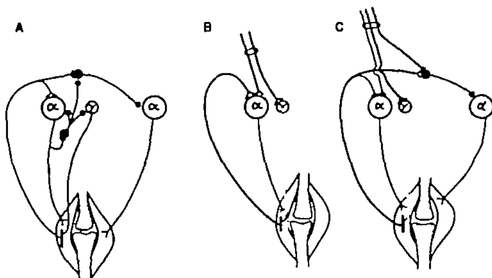


Fig 2.

Schematic representation of neuronal connexions. See text.

will augment motoneuronal firing and thereby add force to the contraction (the load compensating reflex of C. von Euler 1966)

The α-γ-linkage was for a long time discussed only for excitation but Hongo et al (1969) suggested that "essentially the same mechanism operates in the control of reciprocal inhibition" To produce well coordinated movements it seems to be of importance that the reciprocal inhibition of antagonists is coupled to, and coincides with, the excitation of agonists. Since the activation of agonist α-motoneurones often will depend on a spatial facilitation from the "direct" α- and "indirect" γ-routes from a neuronal system (Fig. 2B) it would be advantageous if the reciprocal inhibition also was governed by a similar convergence (Fig 2C, see Hongo et al 1969 Lundberg 1970) It shall be noted that to achieve the same excitation coupling, spatial facilitation must take place somewhere and in the disynaptic Ia inhibitory pathway this can occur only in the interposed inhibitory interneurone.

The concept of "α-γ-linked reciprocal inhibition" reviewed above was based on the findings that numerous neuronal pathways which evoke parallel excitatory effects in α- and γ-motoneurones to an agonist muscle also excite the Ia inhibitory interneurones impinging on motoneurones to its antagonists (see Hongo et al. 1969 Lundberg 1970) It has thus

been established that volleys in the corticospinal tract may cause not only a co-activation of α and γ-motoneurones (Mortimer and Akert 1961 Fidone and Preston 1967 see also Granit 1955 Vedel 1966, cf., however Koeze 1968 and Koeze, Phillips and Sheridan 1968 for work on the baboon) but also a corresponding facilitation of transmission in the Ia inhibitory pathways (Lundberg and Voorhoeve 1962) Activity in the rubrospinal tract excites α-motoneurones to flexors and often also to extensors, although the dominating effects in the latter usually are inhibitory (Hongo et al. 1969) There is still no clear picture of the rubrospinal actions onto γ-motoneurones, but some evidence favours that at least "dynamic" γ-motoneurones to flexors are excited (Appelberg and Kosary 1963 see also Granit 1970 pp 237—238) Hongo et al. (1969) demonstrated that Ia inhibitory transmission to both flexor and extensor motoneurones receives rubrospinal facilitation

The most clearcut example of parallel effects onto α- and γ-motoneurones and Ia inhibitory interneurones is given by the vestibulospinal actions on hindlimb motoneurones (especially those innervating the knee muscles) The vestibulospinal tract thus evokes a monosynaptic excitation in knee extensor α- and "static" γ-motoneurones (Grillner Hongo and Lund 1970 see Grillner 1969b) as well as in the Ia inhibitory interneurones mediating the reciprocal inhibition from the knee extensors to the knee flexor motoneurones (Grillner et al. 1966 Grillner and Hongo 1972) The vestibulospinal reciprocal inhibition in the knee flexors is also efficiently depressed by conditioning volleys in the ventral roots (paper IV) which shows that it is largely mediated via the Ia inhibitory interneurones. Some part of the vestibulospinal reciprocal inhibition is, however conveyed by other interneurones and there is evidence that such a route is the dominant one in the case of the pretibial flexors (Bruggencate, Lundberg and Udo to be published)

A reticulospinal pathway descending in the medial longitudinal fasciculus (MLF) exerts actions onto lumbar motoneurones which are mainly reciprocally organized to those of the vestibulospinal tract (Grillner Hongo and Lund 1968) Stimulation of MLF thus gives rise to a monosynaptic excitation of α and "static" γ-motoneurones supplying flexors. There is also a disynaptic inhibition of knee and ankle extensor motoneurones (Grillner et al. 1968 1971) but this inhibition is not susceptible to recurrent inhibition (paper IV) and the facilitation of transmission in the Ia inhibitory pathway to these motoneurones, when observed, is very weak (Grillner et al. 1971 papers III and IV)

There are now findings which suggest that "α-γ-linked reciprocal inhi-

bition" operates also in spinal reflexes much as described above for the cortico- rubro- and vestibulospinal pathways. This was first demonstrated in the case of the crossed extensor reflex in which a co-activation of α- and γ-motoneurones to extensors occurs (Hunt and Paintal 1958 Grillner 1969a, b) Thus it has recently been revealed that volleys in the contralateral FRA, in parallel with the excitation of extensor motoneurones, facilitate transmission in the Ia inhibitory pathways to flexor motoneurones (Bruggencate et al 1969 Fedina and Hultborn 1972) This implies that the reciprocal inhibition of flexor motoneurones in the crossed extensor reflex may depend on a convergence on Ia inhibitory interneurones from "direct" impulses in the crossed FRA pathway and "indirect" impulses (via the γ-loop) in Ia afferents.

Facilitation of Ia inhibitory transmission from ipsilateral afferents is not only evoked from the FRA pathway but also via a separate pathway from cutaneous afferents (paper II) These findings will now be correlated with available knowledge regarding excitatory actions on α- and γ-motoneurones from the two pathways.

Hagbarth (1952) discovered that pinching the skin above any given muscle excited its α-motoneurones, while stimulation of surrounding areas produced inhibition i. e. demonstrating a pattern different from that of the flexor reflex. It was later demonstrated (Eldred and Hagbarth 1954) that α- and γ-motoneurones were driven in parallel in these characteristic cutaneous reflexes. Not unexpectedly it is found that electrical stimulation of cutaneous nerves, which often supply large skin areas, evokes a mixture of excitation and inhibition in motoneurones (cf paper II) Nevertheless these skin reflexes seem to add another example of parallel effects onto α- and γ-motoneurones and Ia inhibitory interneurones.

Activation of the ipsilateral FRA pathway evokes predominantly excitation of flexor and inhibition of extensor motoneurones (R.M. Eccles and Lundberg 1959) but mixed effects are not uncommon (R.M. Eccles and Lundberg 1959 Holmqvist and Lundberg 1961 Wilson and Kato 1965 paper II) The γ-motoneurones, static as well as dynamic, are usually affected in a corresponding manner although exceptions are seen (for references and a critical evaluation see Grillner 1969b) The facilitation from the ipsilateral FRA of transmission in the Ia inhibitory pathway to extensor motoneurones (paper II) agrees well with the general idea of parallel effects to agonist α- and γ-motoneurones and Ia inhibitory interneurones impinging on antagonist motoneurones. The equally common facilitation of Ia inhibitory transmission to flexor motoneurones is more surprising and there is no clear explanation at hand, although one possi

bility would be that it corresponds to the occasional FRA excitation of extensor motoneurones.

The complex effects evoked from several of the neuronal systems discussed make it difficult to compare in detail the effects on α- and γ-motoneurones and Ia inhibitory interneurones. Further investigations are necessary and should include a simultaneous study of all these parameters in the same preparation Special attention should also be paid to the problem of to what extent the linkage includes both types of γ-motoneurones (cf Matthews 1964 Grillner 1969b Granit 1970) The present situation seems to be that a number of findings favour — whereas there is no strong evidence against — the hypothesis of an α-γ linkage in the reciprocal inhibition" With this conceptual background the facilitation of transmission in the Ia inhibitory pathway from various neuronal systems should perhaps be described in terms of a conjoint excitation of inhibitory interneurones by a "direct route and an "indirect route via the γ-loop

B Reciprocal inhibition in α-movements

A co-activation of α and γ-motoneurones has been described above as the normal feature in several movements. A linked excitation of α- and γ motoneurones has been established even in very fast twitch like movements in man, in which there is hardly any time for the γ-loop to support the excitatory drive on the α-motoneurones (Vallbo 1971) Especially the last observation indicates that the central effects on α and γ-motoneurones are very tightly linked and it can be asked whether a central command for any movement under normal conditions gives a selective excitation of α-motoneurones even under circumstances when a co-activation of γ motoneurones does not play any role in the generation of the movements.

Granit, Holmgren and Merton (1955) found, however that in intercollicularly decerebrate cats with cerebellar ablation or in anemically decerebrated cats the spindles tended to behave like unbiased passive stretch receptors during e. g. neck reflexes which otherwise act in characteristic α γ linkage (Eldred, Granit and Merton 1953 Granit et al. 1955) Under these experimental conditions it seems that the functional link between α- and γ-activation is broken. Subsequent work by Gilman (1969) has, however revealed that the γ-motoneurones in fact are reflexly activated in e. g pinna and neck reflexes also in the latter types of preparation. The functional disorder of the α-γ-linkage under such conditions

seems to depend on a depression of the resting fusimotor discharge (Gilman 1969) with the result that intrafusal muscle contraction in these movements is not sufficient to prevent unloading of the muscle spindles. With the spindles unloaded the load compensating reflex (C. von Euler 1966) cannot work and the whole movement will thus depend on the "direct" excitation of α-motoneurones. Corda, Euler and Lennerstrand (1966) similarly demonstrated that cerebellar stimulation can cause a shift in the balance of α- and γ-drive from a dominating γ-activity (with increasing spindle discharge during the contraction) towards some degree of α-dominance (with decreasing spindle discharge during contraction) or vice versa. It has also been shown that there is a shift towards dominating α-activity in experimental rigidity presumably related to that of human Parkinsonism (Steg 1966). To conclude, although the linked excitation of α- and γ-motoneurones seems to be a normal feature of motor control there are also numerous examples showing that the balance can be shifted in favour of either the α- or γ-route. For the time being and in order to elucidate the mechanisms governing the balance it seems still profitable to distinguish between α-γ-linked movements (with maintained or increased Ia discharge rate) and α-movements (with unloaded spindles).

In papers II and IV (see also Lundberg 1970) reference has been made to the existence of reciprocal inhibition which is not mediated via the Ia inhibitory interneurones. The important question thus arises whether this latter type of inhibition represents the reciprocal inhibition in α movements. Since the α-motoneurones evidently can be fired without the support from the γ-loop it should not be excluded that the same holds true for the Ia inhibitory interneurones, which accordingly might transmit reciprocal inhibition in α-movements as well as in α-γ-linked movements. The selective recurrent depression of Ia IPSPs can be used to differentiate between IPSPs mediated by the Ia inhibitory interneurones and those transmitted via other inhibitory interneurones (papers II and IV) and it will thus be possible to design experiments which can settle this question.

C Recurrent inhibition of "α-γ-linked reciprocal inhibition"

In the previous section the two opposite processes of excitation and reciprocal inhibition were treated rather as a unity although the functional demand on them may be different. If the shortening of a contracting muscle in an α-γ-linked movement is opposed, the load compensating reflex (C. von Euler 1966, see above) will augment the force of con-

traction (since the increased discharge in large muscle spindle afferents produces an increase in motoneuronal firing) The increased Ia discharge also reaches the Ia inhibitory interneurones and will thereby tend to augment reciprocal inhibition The role of inhibition is, however to prevent excitation of motoneurones and inhibition beyond this level may rather be a disadvantage since, if the need should arise, the readiness for activation of these motoneurones would be decreased In summary a command for increased muscular force requires increased excitation of agonistic motoneurones but not necessarily a corresponding increase of reciprocal inhibition.

Since the main recurrent depression of Ia IPSPs is evoked from motor fibres to muscles whose Ia afferents produce the IPSPs (paper V cf also Fig. 2A) Hultborn and Lundberg (1972) suggested that the recurrent control of the Ia inhibitory pathway may have the function of providing a constant depth of reciprocal inhibition during different degrees of agonist activity in movements depending on α-γ-linkage. In a first attempt to test that hypothesis they investigated inhibition of the monosynaptic test reflex to the knee flexors posterior biceps semitendinosus during stretch of the tendon to the knee extensor quadriceps (see Fig. 3A) The discharge in an isolated Ia filament from and the EMG in the quadriceps muscle were also recorded. The principal results are schematically drawn in Fig 3B Since the Ia inhibitory interneurones are spontaneously active in the employed unanesthetized decerebrate preparation (Fedina and Hultborn unpublished, cf Hultborn, Jankowska, Lindström and Roberts 1971) it was not unexpected to find a reciprocal inhibition even with small extensions giving only a slight increase in the Ia firing rate. With increasing extension there is first a linear augmentation of reciprocal inhibition. However when the stretch reflex commences (judged by the EMG) a plateau appears in the inhibition curve although the stretch reflex increases with further extension. This plateau suggests that at different levels of muscle extension, the addition in Ia excitation and recurrent inhibition almost exactly balance each other The results seem compatible with the idea that recurrent inhibition of Ia inhibitory interneurones may have the function of keeping the depth of reciprocal inhibition fairly constant during increased activity of agonist α-motoneurones. Further experiments should, however be designed in which a load compensation does occur and in which the excitation of agonists and the reciprocal inhibition of antagonists are exerted by a neuronal system which gives a co-activation of α- and γ-motoneurones and Ia inhibitory interneurones as indicated by the dashed lines in Fig. 3A. The role

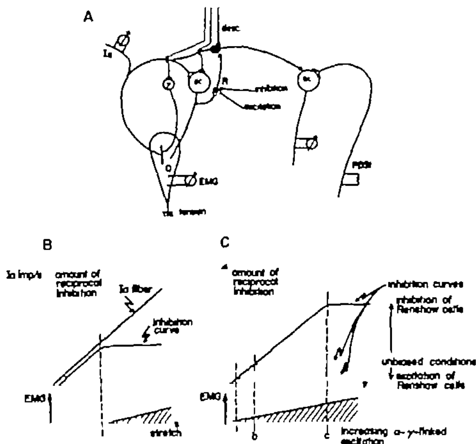


Fig 3

The experimental arrangement is shown in A (quadriceps, Q; posterior biceps and semitendinosus, PBSt; Renshaw cell, R; see further in text). The dashed lines in the top of the drawing indicate the parallel action onto α- and γ-motoneurones of agonists and onto Ia inhibitory interneurones impinging on motoneurones of antagonists as postulated for several descending (desc.) and segmental pathways. Graphs in B show the I impulse frequency of Q afferent and the amount of inhibition f a PBSt monosynaptic test reflex in L7 and S1 entral roots (upper ordinate) and the EMG in the Q muscle (lower ordinate) measured during maintained extension of the Q muscle to increasing lengths (upper and lower abscissa). The dashed vertical line indicates the commencement of the stretch reflex Graphs in C show hypothetical effects of augmented α-γ-linked excitation (i. e. increased activity in an arbitrary pathway represented by the three dotted lines in A; abscissa) on reciprocal inhibition of a monosynaptic test reflex and the EMG in the gonist muscle (ordinates). The expected amount of α-γ-linked reciprocal inhibition is shown for three conditions, () during inhibition of Renshaw cells, (ii) during excitation of Renshaw cells and (iii) during unbiased" conditions. The vertical dashed lines indicate when the excita tory summation from motor axon collaterals is large enough to discharge the Renshaw cells (a, b and c during excitation, "unbiased conditions and inhibition of Renshaw cells respectively see further in text)

of presynaptic inhibition must also be evaluated (cf Hultborn and Lundberg 1972)

The discussion so far has mainly been confined to reciprocal flexion extension movements. However there are also more complex non reciprocal movements, many of which involve a co-contraction of antagonist muscles. In such movements there may not only be a need for a 'constant' reciprocal inhibition during different degrees of agonist contraction (cf above) but also for some mechanism to choose the proper level of inhibition. Sometimes it may even be an advantage if the reciprocal inhibition does not operate at all

Activation of several neuronal systems has been found to evoke parallel effects onto α- and γ-motoneurones and Ia inhibitory interneurones as discussed in section A in General Discussion. It may well be that at least several of these neuronal systems have different subsets, which are capable of independent operation, some exciting α- and/or γ-motoneurones and others Ia inhibitory interneurones. In keeping with this idea Asanuma and Ward (1971) recently found that although intracortical microstimulation in the cat often evoked reciprocal effects this pattern was not obligatory — at several sites stimulation could give excitation of one muscle group without any reciprocal inhibition of antagonists. Since the reciprocal "α-γ-linked inhibition" according to the hypothesis is dependent on the spatial facilitation from "direct" and "indirect" (via the γ-loop) excitation, the withdrawal of the "direct" excitation of the Ia inhibitory interneurones may be enough to prevent their firing (cf Lundberg 1970)

Of more interest in the present connexion is the finding that Renshaw cells are subject to spinal as well supraspinal control (for references see paper V p 661) which can either enhance or depress transmission in the recurrent pathway Hypothetical effects on reciprocal inhibition (mediated by Ia inhibitory interneurones) by facilitation and inhibition of Renshaw cells are illustrated in Fig. 3C. The abscissas in the graph represent increasing α-γ-linked excitation of agonists. On the upper ordinate is the α-γ-linked reciprocal inhibition of antagonists and on the lower ordinate the EMG in the agonist muscle. Let us assume, that under unbiased conditions there is a need for some excitatory summation at Renshaw cell level, i. e. the α-activity to the agonist muscle must reach e. g vertical line b, before the Renshaw cells start to transmit recurrent inhibition. When this level is reached further increments in excitation and recurrent inhibition may balance each other as discussed above. During flexion-extension movements, in which use of effective reciprocal inhibition might be postulated, transmission in the recurrent pathway should be inhibited (cf Fig 3A) This

means that a larger excitation from motor axon collaterals on Renshaw cells is needed in order to overcome the inhibition and allow transmission of recurrent inhibition (vertical line c) It should be recalled that Severin Orlovskii and Shik (1968) in fact have given evidence for a decreased transmission in the recurrent pathway during stepping in which reciprocal inhibition presumably plays an important role (cf Engberg and Lundberg 1969 Lundberg 1969b) Further support may be given by the finding that Renshaw cells are profoundly inhibited (Bergmans, Burke and Lundberg 1969) during activation of neuronal systems of particular interest in relation to alternating flexion-extension movements (Jankowska, Jukes, Lund and Lundberg 1967)

Facilitation of Renshaw cells on the contrary diminishes the need for excitatory summation from motor axon collaterals. Even the slightest activity of agonist α-motoneurones (vertical line a) will give rise to recurrent inhibition of Ia inhibitory interneurones and thereby keep the reciprocal inhibition very small as may be needed in non-reciprocal movements.

Comments on some earlier thoughts on the function of recurrent inhibition of motoneurones

Before closing the discussion some reference will be made also to earlier studies devoted to clarification of the function of recurrent inhibition of motoneurones.

According to Eccles et al (1954) and Eccles, Eccles, Iggo and Ito (1961) the distribution of recurrent inhibition among α-motoneurones is related mainly to their proximity but Eccles et al (1961) also pointed out that not all findings could be explained by this "proximity hypothesis" These authors nevertheless concluded "that the very wide distribution cutting across all functional classification must give recurrent inhibition a general suppressor action on motoneurones of diverse type" In a re-evaluation of the contribution from different nerves to the recurrent inhibition in several species of motoneurones (see paper V) it was instead concluded (i) that motor nuclei to muscles which are linked in Ia synergism are mutually connected by recurrent inhibition regardless of their location in the spinal cord and (ii) that motor nuclei supplying strict antagonists at the same joint do not recurrently inhibit each other even when they have a similar rostro-caudal distribution. Some recurrent inhibition is drawn also from unrelated muscles and these connexions were denoted the "extended pattern

of recurrent inhibition The significance of the latter pattern is unknown and the further discussion will be limited to the recurrent inhibition between motor nuclei linked in Ia synergism.

Granit and Renkin (1961) showed that recurrent inhibition is proportional over a considerable range, to the frequency of antidromic stimulation. Rapidly discharging motoneurones with strong excitatory drive will therefore evoke a more effective inhibition of other neurones in the same motor nucleus which are firing at liminal frequencies, than they themselves suffer from the latter The net result of the recurrent inhibition would thus be a contrast effect concentrating the reflex act to those motoneurones which are at the highest level of excitability and suppressing the contribution from cells at lower levels of excitability" (Granit 1962, p 26) Although this statement is undoubtedly true it remains to be shown whether the suppression of feebly driven motoneurones produces a *spatial* sharpening of the motor response as postulated by Brooks and Wilson (1959 see below)

It is known that group Ia afferents excite monosynaptically not only motoneurones of that muscle (homonymous action) but also motoneurones of synergic muscles (Lloyd 1946b, Laporte and Lloyd 1952 Eccles Eccles and Lundberg 1957b) The latter heteronymous effects are weaker however and the ensuing activity in these motoneurones should therefore be more susceptible to inhibition. An equally strong inhibition of both the homonymous and the heteronymous motoneuronal pools would undoubtedly cause a localization of the motor output. The problem is, however that the autogenous nerve is the main source of recurrent inhibition while volleys in heteronymous nerves give weaker inhibition Accordingly it is not easy to predict the net effect. In an attempt to solve this question experimentally Brooks and Wilson (1959) studied homo- and heteronymous reflexes elicited at high repetition rates (40—175/s) in which a state of continuous recurrent depression is produced. They compared the sizes of these test reflexes before and after administration of dihydro-beta-erythroidine (DHßE, which blocks the activation of Renshaw cells from motor axon collaterals) and found a more pronounced increase of the heteronymous reflexes than of the homonymous, following administration of DHßE. They consequently concluded that the recurrent inhibition plays a part in preservation of the localized stretch reflex. This conclusion however depends on the unsound assumption that the number of motoneurones recruited is linearly related to the amount of additional net excitation in the respective motoneuronal pools, although the sizes of the test reflexes are very dissimilar To allow any conclusions it would be necessary to demon

strate increased heteronymous reflexes also when the stimulus strength of the peripheral nerve is decreased to restore the homonymous test reflex at the same size as before DHβE administration. Therefore, although the idea that *recurrent inhibition of motoneurones causes a spatial sharpening of* the motor response is attractive, it seems at present to lack experimental support.

Recurrent inhibitory effects to α- and γ-motoneurones supplying the same muscle are presumably evoked in parallel (see p. 20). Whatever the function of recurrent inhibition of motoneurones may be, this seems to be a distinct advantage since if the α-motoneurones alone suffered from Renshaw inhibition this would constitute an "internal loading" (see C. von Euler 1970, p. 345) and an optimally biased γ-loop would tend to compensate for this change in α-input as for external loads. This would mean that in order to be effective not only excitation but also inhibition should be evoked in parallel on both α- and γ-motoneurones.

Summary

1 The present studies have aimed at analyzing the excitatory and inhibitory convergence from various neuronal systems onto the inter neurones mediating the reciprocal inhibition of motoneurones from large muscle spindle (Ia) afferents The conclusions regarding the convergence on the Ia inhibitory interneurones rest on studies of how transmission in the Ia inhibitory pathway is influenced from other neuronal systems an excitatory convergence being indicated by facilitation and an inhibitory by depression of the Ia IPSPs The depression of Ia IPSPs by volleys in motor axon collaterals has also been an important tool in the analysis.

2 It is known that Ia afferents from several muscles often can evoke inhibition in a given species of motoneurones. It has now been demonstrated that this convergence, at least partly takes place already at the interneuronal level

3 In spinal cats under chloralose anesthesia Ia IPSPs in flexor as well as extensor motoneurones were regularly facilitated by volleys in cutaneous afferents and high threshold muscle and joint afferents. In decerebrate cats with a low pontine lesion (in which volleys in these afferents evoke inhibition in both extensor and flexor motoneurones) Ia inhibitory transmission was not facilitated but depressed from high threshold muscle and joint afferents. Volleys in low threshold cutaneous afferents, on the other hand, facilitated Ia IPSPs also in this preparation. It is postulated that the Ia inhibitory interneurones receive excitatory actions from the ipsilateral flexor reflex afferents (transmission depressed in the decerebrate state) and through a separate pathway from low threshold cutaneous afferents

4 A bulk of evidence has been gathered demonstrating that excitatory as well as inhibitory actions from various segmental and descending pathways, which control Ia inhibitory transmission, in fact do converge onto the same Ia inhibitory interneurones. Thus the interneuronal pool mediating Ia inhibition to a given motor nucleus can so far be regarded as functionally homogeneous.

5 Whenever conditioning volleys in the ventral root depressed transmission of inhibition to motoneurones from segmental or descending pathways, volleys in these pathways were found to facilitate Ia inhibitory

transmission It was postulated that the recurrent depression of IPSPs in motoneurones indicates to which extent these IPSPs are mediated by the Ia inhibitory interneurones.

6. The investigation of the relative contribution from different efferent nerves to recurrent depression of Ia inhibitory interneurones revealed that the strongest depression is always evoked from motor fibres to muscles whose Ia afferents produce the IPSPs. For example, the Ia IPSP from the knee extensor recorded in motoneurones to a knee flexor is most effectively depressed by antidromic stimulation of motor fibres to the knee extensor The prevailing pattern of recurrent effects indicates that activity in one motor nucleus will cause recurrent inhibition of α and γ-motoneurones within that motor nucleus and other nuclei innervating synergists and also of Ia inhibitory interneurones impinging on motoneurones to antagonists.

7 The convergence onto the Ia inhibitory interneurones was discussed in the light of the recently introduced concept of an "α-γ-linkage in the reciprocal inhibition"

Acknowledgements

I wish to express my deep gratitude to my teacher Professor Anders Lundberg, under whose guidance it has been my privilege to work. His advice and help and the many inspiring discussions have been of greatest value to me during the performance of this work.

I also wish to thank László Fedina, Elzbieta Jankowska, Sivert Lindström, Maurizio Santini and Masao Udo for very stimulating collaboration.

Thanks are also due to Erling Eide with collaborators and Yngve Källström for invaluable technical constructions, to Rauni Larsson for a most excellent assistance in the experiments, to Berit Nilsson and Carina Andersson for typing the various versions of these papers and to the photographer Tomas Palm.

Last but by no means least I would like to thank Kenneth Marshall for scrutinizing the English text in this summary.

This investigation was supported by grants from the Swedish Medical Research Council (Projects No. B70 14X 94-06B and B72 14X 94-08A), Medical Faculty, University of Göteborg ("Främjande av ograduerade forskares vetenskapliga verksamhet") and Svenska Sällskapet för Medicinsk Forskning.

References

Appelberg, B. and I. Z. Kosary, Excitation of flexor fusimotor neurones by electrical stimulation in the red nucleus. *Acta physiol. scand.* 1963 *59* 445—453

Araki, T., J. C. Eccles and M. Ito, Correlation of the inhibitory postsynaptic potential of motoneurones with the latency and time course of inhibition of monosynaptic reflexes. *J. Physiol.* (Lond.) 1960. *154* 354—377

Asanuma, H. and J. E. Ward, Patterns of contraction of distal forelimb muscles produced by intracortical stimulation in cats. *Brain Res.* 1971 *27* 97—109

Baldissera, F., G. ten Bruggencate and A. Lundberg, Rubrospinal monosynaptic connexion with last order interneurones of polysynaptic reflex paths. *Brain Res.* 1971 *27* 390—392.

Bergmans, J., R. Burke and A. Lundberg, Inhibition of transmission in the recurrent inhibitory pathway to motoneurones. *Brain Res.* 1969 *13* 600—602.

Bradley, K. and J. C. Eccles, Analysis of the fast afferent impulses from thigh muscles. *J. Physiol.* (Lond.) 1953 *122* 462—473

Brooks, V. B. and V. J. Wilson, Recurrent inhibition in the cat's spinal cord. *J. Physiol.* (Lond.) 1959 *146.* 380—391

Brown, T. G., On the nature of the fundamental activity of the nervous centres; together with an analysis of the conditioning of rhythmic activity in progression, and a theory of the evolution of function in the nervous system. *J. Physiol.* (Lond.) 1914 *48* 18—46.

Brown, M. C., D. G. Lawrence and P. B. C. Matthews, Antidromic inhibition of presumed fusimotor neurones by repetitive nerve stimulation of the ventral root in the decerebrate cat. *Experientia* 1968. *24* 1210—1211

Bruggencate, G. ten, R. Burke, A. Lundberg and M. Udo, Interaction between the vestibulospinal tract, contralateral flexor reflex afferents and Ia afferents. *Brain Res.* 1969 *14* 529—532.

Burke, R. E., L. Fedina and A. Lundberg, Spatial synaptic distribution of recurrent and group Ia inhibitory systems in cat spinal motoneurones. *J. Physiol.* (Lond.) 1971. *214* 305—326.

Coppin, C. M. L., J. J. B. Jack and A. K. McIntyre, Properties of group I afferent fibres from semitendinosus muscle in the cat. *J. Physiol.* (Lond.) 1969 *203* 45—46P

Coppin, C. M. L., J. J. B. Jack and C. R. MacLennan, A method for the selective electrical activation of tendon organ afferent fibres from the cat soleus muscle. *J. Physiol.* (Lond.) 1970. *210* 18—20P

Corda, M., C. v. Euler and G. Lennerstrand, Reflex and cerebellar influences on α and on "rhythmic" and tonic γ activity in the intercostal muscle. *J. Physiol.* (Lond.) 1966. *184* 898—923

Curtis, D. R., J. W. Phillis and J. C. Watkins, Cholinergic and non-cholinergic transmission in the mammalian spinal cord. *J. Physiol.* (Lond.) 1961 *158* 296—323

Eccles, J. C., *The Physiology of Synapses.* Springer Verlag, Berlin. 1964

Eccles, J. C., P. Fatt and K. Koketsu, Cholinergic and inhibitory synapses in a pathway from motor axon collaterals to motoneurones. *J. Physiol.* (Lond.) 1954 *126.* 524—562.

Eccles, J. C., P. Fatt and S. Landgren, The central pathway for the direct inhibitory action of impulses in the largest afferent nerve fibres to muscle. *J. Neurophysiol.* 1956. *19* 75—98

Eccles, J. C., R. M. Eccles and A. Lundberg, Synaptic actions on motoneurones in relation to the two components of the group I muscle afferent volley. *J. Physiol.* (Lond.) 1957a. *136*. 527—546.

Eccles, J. C., R. M. Eccles and A. Lundberg, The convergence of monosynaptic excitatory afferents on to many different species of α-motoneurones. *J. Physiol.* (Lond.) 1957b. *137*. 22—50.

Eccles, J. C., R. M. Eccles, A. Iggo and M. Ito, Distribution of recurrent inhibition among motoneurones. *J. Physiol.* (Lond.) 1961. *159*. 479—499.

Eccles, R. M. and A. Lundberg, The synaptic linkage of direct inhibition. *Acta physiol. scand.* 1958a. *43*. 204—215.

Eccles, R. M. and A. Lundberg, Integrative patterns of Ia synaptic actions on motoneurones of hip and knee muscles. *J. Physiol.* (Lond.) 1958 b. *144*. 271—298.

Eccles, R. M. and A. Lundberg, Synaptic actions in motoneurones by afferents which may evoke the flexion reflex. *Arch. ital. Biol.* 1959. *97*. 199—221.

Eklund, G. C. von Euler and S. Rutkowski, Spontaneous and reflex activity of intercostal gamma motoneurones. *J. Physiol.* (Lond.) 1964. *171*. 139—163.

Eldred, E., R. Granit and P. A. Merton, Supraspinal control of the muscle spindles and its significance. *J. Physiol.* (Lond.) 1953. *122*. 498—523.

Eldred, E. and K. E. Hagbarth, Facilitation and inhibition of gamma efferents by stimulation of certain skin areas. *J. Neurophysiol.* 1954. *17*. 59—65.

Ellaway, P. H., Antidromic inhibition of fusimotor neurones. *J. Physiol.* (Lond.) 1968. *198*. 39—40P.

Ellaway, P. H., Recurrent inhibition of fusimotor neurones exhibiting background discharges in the decerebrate and the spinal cat. *J. Physiol.* (Lond.) 1971. *216*. 419—439.

Engberg, I., Reflexes to foot muscles in the cat. *Acta physiol. scand.* 1964. *62*. Suppl. 235.

Engberg, I. and A. Lundberg, An electromyographic analysis of muscular activity in the hindlimb of the cat during unrestrained locomotion. *Acta physiol. scand.* 1969. *75*. *614—630*.

Euler, C. v. The control of respiratory movement. In *Breathlessness*. Ed. J. B. L. Howell and E. J. M. Campbell, Blackwell Scientific Publications, Oxford 1966. 19—32.

Euler, C. v. Fusimotor activity in spindle control of natural movements with special reference to respiration. In *Excitatory Synaptic Mechanisms*. Ed. P. Andersen and J. K. S. Jansen, Universitetsforlaget, Oslo, 1970. 341—349.

Fedina, L. and H. Hultborn, Facilitation from contralateral primary afferents of interneuronal transmission in the Ia inhibitory pathway to motoneurones. 1972. In manuscript.

Fidone, S. J. and J. B. Preston, Motor cortex effects on flexor and extensor fusimotor neurons of the cat's hindlimb. *Fed. Proc.* 1967. 26. 657.

Frank, K. and M. G. F. Fuortes, Unitary activity of spinal interneurones of cats. *J. Physiol.* (Lond.) 1956. *131*. 424—435.

Gilman, S., Fusimotor fiber responses in the decerebellate cat. *Brain Res.* 1969. *14*. 218—221.

Golgi, C., La cellula nervosa motrice. In: Opera Omnia. Vol. II, pp. 540—541. Milano: Hoepli 1903.

Granit, R., Reflex self-regulation of muscle contraction and autogenetic inhibition. *J. Neurophysiol.* 1950. *13*. 351—372.

Granit, R., *Receptors and Sensory Perception*. New Haven, Yale University Press, 1955. 369 p.

GRANIT R., Quantitative aspects of control of the discharge frequency of nerve cells. *Proceedings XXII In ernational Physiological Congress*, 1962. *1* pt 1 22—27 Amsterdam Excerpta Medica Foundation.

GRANIT, R., The functional role of the muscle spindle s primary end organs. *Proc R y Soc Med.* 1968 *61* 69—78.

GRANIT R., *The Basis of Motor Control.* Academic Press, London and New York 1970. 1—346.

GRANIT, R., B. HOLMGREN and P A. MERTON The two routes for excitation of muscle and their subservience to the cerebellum. *J Physiol.* (Lond.) 1955 *130* 213—224

GRANIT R. and B. RENKIN, Net depolarization and discharge rate of motoneurones, as measured by recurrent inhibition. *J Physiol.* (Lond.) 1961 *158* 461—475

GRILLNER, S., The fluence of DOPA on the static and the dynamic fusimotor activity to the triceps surae of the spinal cat. *Act physiol scand* 1969 a. *77* 490—509

GRILLNER, S Supraspinal and segmental control of static and dynamic γ-motoneurones in the cat. *Acta physiol. scand.* 1969 b. Suppl. 327 1—34

GRILLNER, S. and T HONGO, Vestibulospinal relations. Vestibular influences on the lumbosacral spinal cord. In *Basic A pects of Central Vestibular Mechanisms.* Eds. A. Brodal and O Pompeiano. Elsevier Publ. Co. 1972. In press.

GRILLNER, S., T HONGO and S. LUND, Interaction between the inhibitory pathways from the Deiters' nucleus and Ia afferents to flexor motoneurones. *Acta physiol. scand.* 1966. *68* Suppl. 277 61

GRILLNER, S T HONGO and S. LUND, Reciprocal effects between two descending bulbospinal systems with monosynaptic connections to spinal motoneurones. *B ain Res.* 1968 *10* 477—480.

GRILLNER, S., T HONGO and S. LUND, The vestibulospinal tract. Effects on alpha-motoneurones in the lumbosacral spinal cord in the cat. *Exp B ain Res* 1970. *10* 94—120.

GRILLNER, S., T HONGO and S. LUND, Convergent effects on alpha motoneurones from the vestibulospinal tract and pathway descending in the medial longitudinal fasciculus. *Exp B ain Res* 1971 *12* 457—479

HAGBARTH, K. E., Excitatory and inhibitory skin areas for flexor and extensor motoneurones. *Acta physiol. scand* 1952. *26.* Suppl. 94 1—58.

HAGBARTH, K. E. and A. B. VALLBO, Discharge characteristics of human muscle afferents during muscle stretch and contraction. *Exp Neurol.* 1968 *22.* 674—694

HAGBARTH, K. E. and A. B. VALLBO, Single unit recordings from muscle nerves in human subjects. *Acta physiol. scand* 1969 *76* 321—334

HOLMQVIST B., Crossed spinal reflex actions evoked by volleys in somatic afferents. *Acta physiol. scand.* 1961 *52.* Suppl. 181 1—67

HOLMQVIST B and A. LUNDBERG, Differential supraspinal control of synaptic actions evoked by volleys in the flexion reflex afferents in alph motoneurones. *A ta physiol. scand.* 1961 *54* Suppl 186. 1—51

HOLMQVIST B., A. LUNDBERG and O OSCARSSON, Supraspinal inhibitory control of trans mission to three ascending pathways influenced by the flexion reflex afferents. *Arch ital. Biol.* 1960 *98* 60—80.

HONGO, T E. JANKOWSKA and A. LUND ERG, Convergence of excitatory and inhibitory action on interneurones in the lumbosacral cord. *Exp B ain Res* 1966. *1* 338—358

H NGO, T E. JANKOWSKA and A. LUNDBERG, The rubrospinal tract. II. Facilitation of interneuronal transmission in reflex paths to motoneurones. *Exp Brain Res.* 1969 *7* 365—391

HULTBORN, H. and A. LUND ERG, Reciprocal inhibition during the stretch reflex. *Acta physiol scand.* 1972. In press.

HULTBORN H. and M. SANTINI Supraspinal control f monosynaptically activated group Ia interneurones in the ventral horn. *Acta physiol. scand.* 1972. *84* 142—144

HULTBORN, H., E. JANKOWSKA and S. LINDSTRÖM, Recurrent inhibition from motor axon collaterals of transmission in the Ia inhibitory pathway to motoneurones. *J Physiol.* (Lond.) 1971 a. *215* 591—612.

HULTBORN, H., E. JANKOWSKA and S. LINDSTRÖM Recurrent inhibition of interneurones monosynaptically activated from group Ia afferents. *J Physiol.* (Lond.) 1971 b. *215* 613—636.

HULTBORN, H., E. JANKOWSKA, S. LINDSTRÖM and W ROBERTS, Neuronal pathway of the recurrent facilitation of motoneurones. *J Physiol* (Lond.) 1971. *218* 495—514

HUNT C. C., The effect of stretch receptors from muscle on the discharge of motoneurones. *J Physiol.* (Lond.) 1952. *117* 359—379

HUNT C. C. and S. W KUFFLER, Further study of efferent small-nerve fibres to mammalian muscle spindles. Multiple spindle innervation and activity during contraction. *J Physiol.* (Lond.) 1951 a. *113* 283—297

HUNT C. C. and S. W KUFFLER, Stretch receptor discharges during muscle contraction. *J Physiol.* (Lond.) 1951 b. *113* 298—315

HUNT C. C. and A. S. PAINTAL, Spinal reflex regulation of fusimotor neurones. *J Physiol.* (Lond.) 1958 *143* 195—212.

JANKOWSKA, E. and W ROBERTS, Function of single interneurones established by their monosynaptic inhibitory effects on motoneurones. *Acta physiol. scand.* 1971 *82* 24A—25A.

JANKOWSKA, E. and W J ROBERTS, An electrophysiological demonstration f the axonal projections of single spinal interneurones in the cat. *J Physiol.* (Lond.) 1972 a. In press.

JANKOWSKA, E. and W J ROBERTS, Synaptic actions of single interneurones mediating reciprocal Ia inhibition of motoneurones. *J Physiol.* (Lond.) 1972 b. In press.

JANKOWSKA, E., M. G M JUKES, S. LUND and A. LUNDBERG, The effect of DOPA on the spinal cord. 5 Reciprocal organization of pathways transmitting excitatory action to alpha motoneurones of flexors and extensors. *Acta physiol. scand.* 1967 *70* 369—388

KOEZE, T H., The independence of corticomotoneuronal and fusimotor pathways in the production of muscle contraction by motor cortex stimulation. *J Physiol.* (Lond.) 1968 *197* 87—105

KOEZE, T H., C. G. PHILLIPS and J D SHERIDAN, Thresholds of cortical activation f muscle spindles and motoneurones of the baboon's hand. *J Physiol.* (Lond.) 1968 *195* 419—449

LAPORTE, Y and P BESSOU Étude des sousgroupes lent et rapide d groupe I (fibres afférentes d origine musculaire de grand diamètre) chez le chat. *J Physiol.* (Paris) 1957 *49* 1025—1037

LAPORTE, Y and D P C. LLOYD, Nature and significance f the reflex connections established by large afferent fibres of muscular origin. *Amer J Physiol.* 1952. *169* 609—621

LEKSELL, L., Th action potential and excitatory effects of the small entral root fibres to skeletal muscle. *Acta physiol scand.* 1945 *10* Suppl. 31 1—84

LIDDELL, E. G. T and C S. SHERRINGTON, Reflexes in response to stretch. *Proc R y Soc* 1924 *96B* 212—242.

LIDDELL, E. G. T and C. S. SHERRINGTON Further observations on myotatic reflexes. *Proc Roy Soc B* 1925. *97* 267—283

LLOYD, D P C., A direct central inhibitory action f dromically conducted impulses. *J Neurophysiol.* 1941 *4* 184—190.

LLOYD, D P C., Reflex action in relation to pattern and peripheral source of afferent stimulation. *J Neurophysiol.* 1943 a. *6* 111—120.

Lloyd, D P C, Neuron patterns controlling transmission of ipsilateral hind limb reflexes in cat. *J Neurophysiol* 1943 b. *6*, 293—315

Lloyd, D. P C, Conduction and synaptic transmission of reflex response to stretch in spinal cats. *J Neurophysiol* 1943 c. *6* 317—326.

Lloyd, D P C, Facilitation and inhibition of spinal motoneurons. *J Neurophysiol.* 1946 a. *9* 421—438

Lloyd, D P C, Integrative pattern of excitation and inhibition in two-neuron reflex arcs. *J Neurophysiol* 1946 b. *9* 439—444

Lundberg, A., Convergence of excitatory and inhibitory action on interneurones in the spinal cord. In *The Interneuron* Ed. M. A B. BRAZIER, UCLA Forum Med. Sci., No. 11 University of California Press, Los Angeles. 1969 a. 231—265

Lundberg, A., *Reflex control of stepping* The Nansen Memorial Lecture V Universitetsforlaget, Oslo 1969 b

Lundberg, A., The excitatory control of the Ia inhibitory pathway. In *Excitatory Synaptic Mechanisms*. Ed. P Andersen and J K. S. Jansen. Universitetsforlaget, Oslo 1970. 333—340.

Lundberg, A. and P Voorhoeve, Effects from the pyramidal tract on spinal reflex arcs. *Acta physiol scand* 1962. *56*. 201—219

Lundberg, A and G Winsbury Selective adequate activation of large afferents from muscle spindles and Golgi tendon organs. *Acta physiol. scand* 1960. *49* 155—164

Matthews, P B. C, Muscle spindles and their motor control. *Physiol. Rev* 1964 *44* 219—288.

Merton, P A., Speculations on the servo-control of movement. In *The Spinal Cord* Ed. Wolstenholme. Churchill Ltd., London 1953

Mortimer, E. M. and K. Akert Cortical control and representation of fusimotor neurons. *Amer J Phys. Med* 1961 *40*. 228—248

Noth, J Recurrente Hemmung der Extensor Fusimotoneurone? *Pflügers Arch*. 1971 *329* 23—33

Prestige, M. C, Initial collaterals of motor axons within the spinal cord of the cat. *J comp Neurol*. 1966. *126* 123—136.

Ramón y Cajal, S., *Histologie du système nerveux de l'homme et des vertébrés*. 1909 *1* Paris Maloine.

Renshaw B., Influence of the discharge of motoneurons upon excitation of neighboring motoneurons. *J Neurophysiol*. 1941 *4* 167—183

Renshaw B., Central effects of centripetal impulses in axons of spinal ventral roots. *J Neurophysiol*. 1946 *9* 191—204

Ryall, R. W., Renshaw cell mediated inhibition of Renshaw cells: Patterns of excitation and inhibition from impulses in motor axon collaterals. *J Neurophysiol*. 1970. *33* 257—270.

Ryall, R. W M. F Piercey and C. Polosa, Intersegmental and intrasegmental distribution of mutual inhibition of Renshaw cells. *J Neurophysiol*. 1971. *34* 700—707

Scheibel, M. E. and A. B. Scheibel, Inhibition and the Renshaw cell. A structural critique. *Brain Behav Evol*. 1971 *4* 53—93

Sears, T A., Efferent discharges in alpha and fusimotor fibres of intercostal nerves of the cat. *J Physiol*. (Lond.) 1964 *174* 295—315

Severin, F V G N Orlovskii and M. L. Shik, Work of the muscle receptors during controlled locomotion. *Biophysics* 1967 *12* 575—586. (Eng. transl. of *Biofizika* 1967 *12* 502—511)

Severin, F V G N Orlovskii and M. L. Shik, Recurrent inhibitory effects on single motoneurones during an electrically evoked locomotion. (In Russian) *Bull exp Biologii Medicìny* 1968 66 3—9

Steg, G Efferent muscle control in rigidity In *Muscular Afferents and Motor Control.* Nobel Symposium I. Ed R. Granit. Almqvist & Wiksell, Stockholm 1966. 437—443

Sumner, A Properties of Ia and Ib afferent fibres serving stretch receptors of the cat's medial gastrocnemius muscle. *Proc Univ Otago med School* 1961 39 3—5

Szentágothai, J Synaptic architecture of the spinal motoneuron pool; in Recent advances in clinical neurophysiology *Electroenceph clin. Neurophysiol.* 1967 Suppl. 25 pp 4—19 Elsevier Publ. Co., Amsterdam.

Taylor, A. and M. R. Davey Behaviour of jaw muscle stretch receptors during active and passive movements in the cat. *Nature* 1968 220 301—302.

Vallbo, A. B., Muscle spindle response at the onset of isometric voluntary contractions in man. Time difference between fusimotor and skeletomotor effects. *J Physiol.* (Lond.) 1971 218 405—431

Vedel, J P Mise en évidence d un contrôle cortical de l activité des fibres fusimotrices dynamiques chez le Chat par la voie pyramidale C R. Acad Sc Paris 1966. 262 908—911

Wilson, V J Recurrent facilitation of spinal reflexes. *J gen Physiol.* 1959 42 703—713

Wilson, V J and P R. Burgess, Disinhibition in the cat spinal cord. *J Neurophysiol.* 1962. 25 392—404

Wilson, V J and M. Kato, Excitation of extensor motoneurons by group II afferent fibers in ipsilateral muscle nerves. *J Neurophysiol.* 1965 28 545—554

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 376

DRUG-INDUCED CHANGES IN THE RELEASE AND UPTAKE OF BIOGENIC AMINES

A STUDY ON MAST CELLS

BY
MARIANNE FRISK HOLMBERG

STOCKHOLM 1972

AB Tryckmans, Stockholm 972

Contents

The present survey is based on studies carried out at the Department of Pharmacology Karolinska Institutet, Stockholm and summarizes the results presented in the following publications

I The mechanism of histamine release from isolated rat peritoneal mast cells induced by d tubocurarine. *Acta physiol scand* 1969 *76* 335—339 (Together with B Uvnas)

II Histamine release from rat peritoneal mast cells and cat paws induced by some neuromuscular blocking agents. *Acta physiol scand* 1971 *81* 367—375 (Together with K. Strandberg)

III On the mechanism of chlorpromazine-induced histamine release from rat mast cells. *Acta physiol scand* 1971 *83* 412—421

IV The relationship between lipophilic nature of tricyclic neuroleptics and antidepressants, and histamine release *European J Pharmacol* 1972 In press. (Together with E. v der Kleijn)

V The influence of chlorpromazine on the uptake of biogenic amines by rat mast cells *in vitro* *Acta physiol scand* 1972 In press. (Together with B Uvnäs)

VI. Inhibition of the uptake of biogenic amines into mast cells by tricyclic psychoactive drugs. *Acta physiol scand* 1972 In press.

These papers will be referred to by their Roman numerals.

I Introduction

Biogenic amines, such as 5 hydroxytryptamine (5 HT) dopamine (DA) noradrenaline (NA) adrenaline (A) and histamine (Hi) are stored in granules in for example mast cells, chromaffin cells, enterochromaffin cells, thrombocytes and basophil leucocytes.

It has long been known that different agents, including certain drugs, can release these amines from cellular stores and/or affect their uptake into cells (see Mechanisms of Release of Biogenic Amines, 1966) Release of Hi from mast cells is thought to play an important role in the pathogenesis of ana phylatic shock and other hypersensitivity reactions (Dragstedt and Gebaur Fuelnegg 1933 Mongar and Schild 1962 Mota 1963) Such reactions have been reported to occur during treatment with certain drugs, for example d-tubocurarine (Paton 1959 Westgate and van Bergen 1962, Harrison 1966 Salem *et al* 1968 Hainsworth and Bingham 1970, Takki and Tannisto 1971) and chlorpromazine (Hollister 1958 Anselmo 1963 Meyer 1966 Pfeiffer *et al* 1969 Schwartz 1969)

In studies I III and V the mechanisms by which d tubocurarine and chlorpromazine release Hi from and influence the uptake of amines into mast cells were investigated. In addition, structurally related drugs were studied (II IV and VI) to ascertain if there was any correlation between their chemical structures and physical properties on the one hand and their amine-releasing effects and influence on amine uptake on the other

II Methods

1 Experimental techniques

The investigations were carried out on rat mast cells *in vitro* The release studies[1] were also performed on mast cell rich cat tissue *in situ*
In vitro techniques offer serveral advantages. Thus it is possible to use media which are well defined with respect to pH, ion composition and temperature and to study the influence of these factors on the effects of the drugs. In addition, it was considered valuable to study the effects of the drugs on *in situ* preparations to obtain information about possible qualitative differences in release between *in vitro* and *in situ* preparations.

Isolation of rat mast cells from peritoneal and pleural cavities was carried out according to Uvnäs and Thon (1959) and perfusion of cat paws according to Strandberg (1971) In order to eliminate possible effects of the isolation procedure on the reactivity of the cells the release studies were also performed on mixed cell suspensions[2] from the peritoneal cavity No qualitative differences were found between the results of experiments with isolated cells and those with mixed cells. The methods used have been described in detail in the individual publications.

a) *Studies on rat mast cells*

The peritoneal and pleural cavities of male Sprague-Dawley rats were flushed with a physiological salt solution (NaCl 154 mM KCl 2 7 mM, $CaCl_2$ 0 9 mM, containing 10 % Sörensen s phosphate buffer (Na_2HPO_4+ KH_2PO_4 67 mM) or 70 mM Tris buffer) pH 6—7 0 Isolation of the cells was carried out using a density gradient of the polysaccharide Ficoll (Uvnäs and Thon 1959)

In the release studies the cells were incubated under different conditions

[1] Only Hi-release was studied, since other investigations ha show that 5-HT released at the same time as Hi from rat mast cells and mast cell-rich cat tissues (Moran *et al.* 962 Westerholm 964).
[2] Mast cells are the only Hi-containing cells a cell suspension from the peritoneal cav ty (Smith 958 Lagunoff and Benditt 960).

with the drug and the release process interrupted by instantaneous cooling followed by centrifugation The percentage release or the quantity of amine released was then determined (I II III and IV)

In the uptake studies the influence of the drugs on the uptake of ^{14}C — 5 HT DA, NA and Hi was investigated under different conditions. In addition the uptake of ^{35}S-chlorpromazine was studied. After interrupting the uptake by cooling and centrifuging the radioactivity in the incubation solution was washed from the cells and the cells then disrupted in distilled water The uptake was then measured Under these experimental conditions the spontaneous amine release was 15 ± 3.6 % (n = 42) No metabolites of the amines taken up could be demonstrated (V and VI)

b) *Studies on cat paws*

The main artery of each paw was perfused with physiological salt solution (see above) until the perfusate was free from blood. Each paw was then mounted in a temperature-controlled chamber and perfused at a constant rate of 1 ml/min with the aid of an infusion pump The perfusate was collected continuously during 20 min periods in ice-cooled tubes, according to the method described by Strandberg (1971) and in study II Drugs were added to the perfusion fluid and infused after a 20 min control period.

c) *Morphological studies* were carried out using either light microscopy or electron microscopy (III) the latter by Docent T Hökfelt of the Dept. of Histology Karolinska Institutet.

2 Analytical techniques

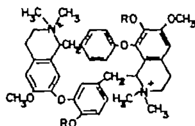
Partition ratios

Determination of the partition coefficients of tricyclic psychoactive drugs was carried out by shaking an aqueous phase, buffered to different pHs, with either chloroform or octanol The drugs were dissolved in either the aqueous phase or the organic phase and the concentrations in the two phases were measured spectrophotometrically after equilibration. The apparent and true partition coefficients were then calculated (IV)

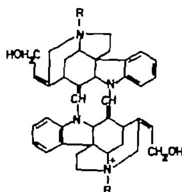
Sodium and potassium ions in mast cells were measured by flame photometry

Lactic dehydrogenase (LDH) was determined using the technique described by Diamant (1967)

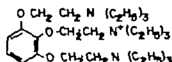
NONDEPOLARIZING AGENTS



	R
TUBOCURARINE	H
DIMETHYL TC	CH_3



	R
C TOXIFERINE	CH_3
ALCURONIUM	$CH_2CH\ CH_2$

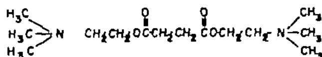


GALLAMINE

DEPOLARIZING AGENTS



DECAMETHONIUM



SUXAMETHONIUM

Table I. *Structural formulae of neuromuscular blocking drugs used*



NEUROLEPTICS

	10	PH OTHIAZINES
PROMET Z NE		CH_2 C $N(CH_3)_2$ CH_3
OM Z E		$-CH_2-CH_2-CH_2-N(CH_3)_2$
C LO P O Z E	C	$-CH_2-CH_2-CH_2-N\ CH_3)_2$
LE OME OM ZI E	OC 3	2-C 2 C 3 C 3) CH_3
		P E ZI E SI E C N
P OC LOE Z E	C	CH_2 C 2-C 2 N N CH_3
T UO E Z E	C 3	C C 2 C 2 N N C 3
E Z E	C	N $CH_2-CH_2-CH_2$ N N-CH $-CH_2OH$
LU E Z E	C 3	$-CH_2-CH_2-CH_2$ N $N-CH_2-CH_2OH$
CET E ZI E	$COCH_3$	$-CH_2-CH_2-CH_2$ N $N-CH_2-CH_2OH$
		P E S E C N
ORID Z E	SC 3	-C 2 C 2 N CH_3
		E S
C O P O E E	C	C $=CH_2-CH_2-CH_2-N\ CH_3)_2$
C O O		C $=CH_2-CH-CH_2$ N N CH_2CH_2OH
	3	C $CH_2-CH-CH_2$ N N-CH CH_2OH



ANTIDEPRESSANTS

	10		R_2
M T T	$-CH_2-CH_2$		$=CH-CH_2-CH_2-N(CH$
NO	$-CH_2-CH_2$	C	$=CH-CH_2-CH_2$ N H CH_3
PROTR TY E	$-CH=CH-$		-CH $-CH_2-CH_2-CH_2$ N H CH_3
MI RAMI	$-CH_2-CH_2$		-CH $-CH_2-CH_2-N(CH_3)$
LO MIPR	$-CH_2-CH_2-$		$-CH_2-CH_2-CH_2-N\ CH_3)$ Cl
PR M T	-CH $-CH_3$	N	$-CH_2-CH-CH_2-N$ H CH_3

T ble II Structu l formulae f tricyclic psychoactive drugs used

Hi was assayed fluorimetrically by the method described by Shore *et al* (1959)
ATP-hydrolysis was determined fluorimetrically according to the method used by Diamant and Peterson (1970)

Liquid scintillation counting

The radioactivity in wash fluids and in mast cells was measured using liquid scintillation spectrometry

3 Statistical methods

In the evaluation of the significance of the results the Student's t test was used for paired comparisons and analysis of variance for comparisons within groups.

III Release studies

1 Release studies on mast cells

Paton (1957) divided the substances that release Hi into several classes. Of these, histamine liberators are as a rule basic compounds which cause Hi release from the tissues on the first exposure.

In connection with studies of Hi release from mast cells in different tissues it has been observed using light microscopy that granules leave the cell i.e. degranulation occurs (Riley 1953 1959 Riley and West 1966) The findings have later been confirmed by electron microscopic studies on rat mast cells (Smith and Lewis 1957 Bloom and Haegermark 1965 Röhlich *et al* 1971) On the basis of these observations, it was considered that in general amine release does not occur without simultaneous degranulation. Biochemical studies have shown that degranulation induced by polymeric compounds, of which compound 48/80 is the most extensively studied, is dependent on endogenous cellular energy production this is not the case with monomers such as alkylamines (Moran *et al* 1962). However there are certain species differences with regard to the energy dependence of the release process (Green 1962, Selye 1965)

Rat mast cells normally contain Hi and 5 HT (Benditt *et al* 1955 Padawer and Gordon 1955 Moran *et al* 1962) The cells can release these amines *in vitro* and *in vivo* (Paton 1957 Rothschild 1966) and take up not only Hi and 5 HT but also NA and DA (Green 1962, 1966 Enerbäck and Häggendal 1970) Mast cells can also store all the above amines in their granules after uptake *in vitro* (Bergendorff and Uvnäs 1967) Furthermore they can produce Hi, 5 HT and DA *in vitro* by decarboxylating the corresponding amino acids (Slorach and Uvnäs 1968)

Hi and 5 HT are stored in mast cells in membrane-bound basophil granules. Apart from the amines, these granules consist mainly of protein and heparin (Lagunoff *et al.* 1964) On a molar basis, the Hi content is 10—30 times the 5 HT content (Moran *et al.* 1962) Different possible modes of binding of the amines in the granules have been discussed (Green 1966) and there are studies which suggest that the amines are electrosta

tically bound (Lagunoff *et al* 1964, Thon and Uvnäs 1966) mainly to carboxyl groups in the granular protein-heparin complex (Uvnäs and Thon 1966 Uvnäs *et al* 1970)

The amines are released from isolated membrane-free granules *in vitro* by ion exchange with cations e.g. sodium. This release process does not require energy (Uvnäs *et al* 1970) Granules released from mast cells (Bloom and Haegermark 1965 Rohlich *et al* 1971) are devoid of a surrounding membrane. Thus amine release from the binding sites is a passive process, while degranulation is considered to be the energy requiring step (Thon and Uvnäs 1967 Uvnäs 1968)

Substances which induce amine release even when cellular energy production is blocked are believed to do so by damaging the cell-membrane (Rothschild 1966 Uvnäs 1968) In such cases the amine release could also be due to contact between the granules and intra and extracellular cations. It has been observed (Bloom and Haegermark 1965) that when mast cells are treated with compound 48/80 granules do not always leave the mast cell in proportion to amine release and for this reason an intracellular release mechanism has been proposed (Carlsson and Ritzén 1969) Even the Hi release induced by the phosphoric acid ester ATP is thought to be energy dependent, but whether or not this release is coupled to the release of granules from the cell is not yet clear (Bloom *et al* 1970) Against the background of these results it is questionable if it is necessary for the granules to leave the cell for amine release to take place.

However these different views can, at least as far as compound 48/80 is concerned, be reconciled by the electron microscopic investigations in which it has been shown that the granules which do not leave the cell in connection with the amine release are in communication with the extracellular space. These granules can therefore be considered to be biologically extracellular and the same release mechanism could therefore be applicable (Röhlich *et al* 1971)

2 Release mechanism for d-tubocurarine and certain structurally related drugs

Investigations have shown that d-tubocurarine induces Hi release through an energy-dependent process (Rocha E Silva and Schild 1949 Rothschild 1966) d Tubocurarine at a concentration of 10^{-4} M released Hi from mast cells (I, II) The release was pH-dependent (with maximal release at pH 7.2) dependent on endogenous cellular energy production and stimulated

by Ca^{++} (1 mM). During the release basophil granules were expelled. Analogous results (II) were obtained on perfused cat paws and therefore it seems likely that d-tubocurarine induces Hi release in the same way in both these preparations. Compound 48/80-induced amine release is stimulated by Ca^{++} and is energy-dependent in both these species (Uvnäs 1966, Strandberg 1971) in the same way as release induced by d-tubocurarine. This supports the view that the same release mechanism is operating for these compounds, and it is thus sensitive to both monomeric and polymeric compounds. The importance of Ca^{++} for the secretion of biologically-active substances, such as hormones and biogenic amines, is well known. It has been proposed that Ca^{++} play an important role in the process of exocytosis (Douglas 1968). A similar process ("sequential exocytosis") has been described in connection with compound 48/80 treatment of mast cells (Röhlich *et al.* 1971).

Detailed investigations have been carried out in order to clarify the importance of the drug molecule's structure for its neuromuscular blocking activity *in vitro*. Of the non-depolarizing drugs (see Table I) dimethyl-d-tubocurarine is more active than d-tubocurarine and the semi-synthetic toxiferines are the most active (Waser 1966, Koelle 1970). Factors such as the molecule's steric shape and its lipophilic and hydrophilic properties are thought to be of importance for its neuromuscular blocking activity *in vitro* and *in vivo* (Cavallito 1967).

The Hi releasing activity of the drugs listed in Table I was studied (II) in order to ascertain if there was any relation between this property and the chemical structures of the drugs. Depolarizing compounds ($\leq 5 \cdot 10^{-3}$ M) lacked releasing activity. Among the non-depolarizing compounds ($>10^{-4}$ M $\leq 5 \cdot 10^{-3}$ M) the curarines were more active than the toxiferines.

The distances between the nitrogen groups in the curare (12.5 Å) and toxiferine (14.5 Å) molecules are different (Schmid 1967, Koelle 1970). However, the likelihood that this difference is of importance for the releasing activity appears small. The aliphatic diamines suxamethonium and decamethonium, *in which the distance between the nitrogen groups is 12—14 Å* (Koelle 1970), have in fact no Hi releasing effect. This implies that other properties of the drug's structure are important for its Hi releasing activity and also that there is no connection between this activity and neuromuscular blocking activity.

3 Release mechanism for chlorpromazine and certain structurally-related drugs

CHLORPROMAZINE

Chlorpromazine's influence on the amine content of cells has been investigated in several studies for example, Hi and 5 HT release from thrombocytes (Bartolini *et al* 1961 Paasonen 1964 Athee 1966 Tuomisto 1968) A release from adrenals (Vapaatalo 1968) and 5 HT release from mast cells (Jansson 1970) have been studied. In these investigations it has been found that the amine release is not energy-dependent. In morphological studies on rat mast cells, granules have been observed extracellularly in connection with Hi (Högberg and Uvnäs 1957 Le Blanc 1958) and 5 HT (Jansson 1970) release.

In study III the release mechanism induced by chlorpromazine was studied on rat mast cells The results showed that chlorpromazine ($>10^{-5}$ M) released histamine and changed the permeability of the mast cell membrane. The change in permeability led to an increase in the cellular Na^+ content and a reduction in the Hi and K^+ contents There was a quantitative relation between the increase in cellular Na^+ and the quantity of Hi released. The cellular content of LDH decreased at higher chlorpromazine concentration ($>5 \cdot 10^{-5}$ M), suggesting that cell damage was occuring In accordance with the above-mentioned investigations Hi release was not influenced by metabolic and enzymatic inhibitors.

Release of amines from the granule binding sites appeared to take place with the granules still *in situ* However when membrane and cell damage occurred granules were observed extracellularly (III): these probably left the cell through the damaged membrane. This would also explain the above-mentioned reports (Högberg and Uvnäs 1957 Jansson 1970) The latter investigations had in fact been carried out with such high concentrations of chlorpromazine that membrane damage almost certainly occurred

Several observations support the concept that Na^+ played an important role in the release of Hi from its binding sites. For example, both the increase in cellular Na^+ and the release of Hi were counteracted by tetrodotoxin[1] (0.3—3 μM) Chlorpromazine did not appear to participate in the granular ion-exchange since on a molar basis the uptake of ^{35}S-chlorpromazine was about a fiftieth of the Hi release and, in addition, chlorpromazine is probably bound to other groups in the granules than carboxyl groups in the protein heparin complex (Popova *et al.* 1971)

Chlorpromazine (50 μg/ml—200 μg/ml) also released Hi when infused

[1] A fish poison which prevents Na^+ influx into cells (Kao 1966).

into cat paws, the threshold concentration was 100 μg/ml Furthermore, Hi release was greater in a Ca^{++} free medium than in the presence of 5 0 mM Ca^{++} (unpublished) These results indicate that chlorpromazine-induced Hi release from rat mast cells and cat paws take place under analogous conditions, which suggests that the release mechanisms are similar

PHENOTHIAZINE AND THIOXANTHENE DERIVATIVES AND TRICYCLIC ANTIDEPRESSANTS

The Hi releasing activity of analogues of chlorpromazine and tricyclic antidepressants (see Table II) has been investigated on rat mast cells (IV). The dose-response curves were parallel for all the drugs studied, which suggests that the release mechanisms induced were similar

The following changes in the drug s structure were shown to be of importance for the releasing activity:

a. Halogen groups (CF_3 Cl) in the tricyclic ring system increased amine release, whilst acetyl groups had the opposite effect.
b A side chain at position 10 in the tricyclic ring system appeared to be necessary for releasing activity Compounds without such substitution were inactive (phenothiazine and chlorphenothiazine) The side chain s structure influenced release quantitatively — it increased if ring systems (piperidine or piperazine), double bonds or terminal methyl groups were present in the side chain and decreased if terminal alcohol groups were introduced or if the chain was branched. Compounds with monomethylated side chains were less active than their dimethylated homologues.

When cat paws were perfused with trifluoperazine, chlorpromazine and promazine the threshold concentrations for Hi release were 50 μg/ml, 100 μg/ml and 200 μg/ml respectively Thus trifluoperazine was more active than chlorpromazine, which in turn was more active than promazine (unpublished) This is analogous with the results obtained using mast cells.

The importance of the drug s lipophilic properties

Alkalinization of the incubation medium increased chlorpromazine s amine releasing potency (III) Since phenothiazine derivatives and tricyclic antidepressants have strong lipophilic properties and pKa values between 8—11 (Green 1967 Domino *et al.* 1968) these properties are probably important for the amine releasing activity However no correlation between the dissociation constants and the biological activities of the drugs

Cat paws were perfused for 4×10 min with physiological salt solution (see Methods) pH 7.0 containing mM EDTA and chlorpromazine (00 μg/ml) and then for 4× min with the same perfusion medi m with the ddition of 5 mM Ca^{+}

could be demonstrated (Green 1967) On the other hand other investigations have suggested that a relation exists between the lipid solubility of aromatic amines and their ability to release biogenic amines e.g Hi from rat mast cells (Rothschild 1966) and 5 HT from thrombocytes (May *et al* 1967)

The importance of the lipophilic properties for the amine releasing activity of tricyclic psychoactive drugs was studied A measure of the lipophilic properties of the drugs was obtained by determining their partition coefficients (IV) The studies showed that the compounds that were more markedly lipophilic were more active Hi releasers than less lipophilic compounds. There was a significant correlation between the apparent partition coefficients at the incubation pH and the Hi releasing activities. There was no corresponding correlation between the true partition coefficient (i e. for the undissociated form) for these tricyclic psychoactive drugs and amine release, which implies that the degree of dissociation is also of importance for the amine releasing activity

In vitro investigations in which amine release from thrombocytes (Bartolini *et al* 1961 Athee 1966 Tuomisto 1968) brain tissue (Carlsson *et al* 1969) and adrenals (Vapaatalo *et al* 1966) has been studied have shown that halogen substituted derivatives and derivatives with piperazine or piperidine side chains are more active than unsubstituted homologues. The same order of activity was found in the investigation on mast cells (IV) In addition it has been shown using thrombocytes that the monomethylated compounds desipramine and nortriptyline are more active amine releasers than the dimethylated homologues imipramine and amitriptyline (Athee 1966 Athee and Paasonen 1968) The reason for this difference may be that plasma protein was present in the latter investigations but not in study IV Phenothiazine derivatives and antidepressants are to a large extent (90—99 %), bound to plasma protein (Borgå *et al* 1969 Curry *et al* 1970) but differences in the degree of protein binding have been demonstrated. Dimethylated antidepressants are bound to a greater (about 1 3 times) extent to plasma protein than are the monomethylated compounds (Borgå *et al* 1969 Glasser and Kriegelstein 1970) The *in vitro* cell lysing effect of tricyclic psychoactive drugs on erythrocytes (Seeman 1966 Athee 1966 Domino *et al* 1968 Baur 1971) and liver cells (Zimmerman and Kendler 1970) also shows a similar dependence on the drug s structure. This implies that these drugs produce a general effect on membranes which leads to irreversible membrane damage at increased drug concentrations Even though the lipophilic nature appears to determine the biological activity the mechanism of action is not necessarily directly dependent on this property

Comparative clinical studies of tricyclic psychoactive drugs, with regard to their neuroleptic and antidepressant effects, are difficult to evaluate, since it is hard to find standardised clinical parameters. However phenothiazine derivatives with a piperazine or piperidine ring in the side chain especially derivatives with halogen groups in the tricyclic ring system are considered to have more potent neuroleptic effects than the corresponding unsubstituted derivatives (Lingjaerde 1970, Cole and Davis 1968) The same order of potency also exists regarding the amine releasing effect *in vitro* (see part III:3 IV) which suggests that the drug's lipophilic properties are of importance for its neuroleptic effects *in vivo*

It has not been possible to demonstrate with certainty any difference in the antidepressant effect of various tricyclic antidepressants (Lingjaerde 1970, Davis *et al* 1968) However the clinical action of these drugs may be divided into several components, for example mood-elevating and psychomotor activating (Kielholz and Pöldinger 1968) Among the antidepressant drugs, tertiary amines such as imipramine and amitriptyline are considered to be more effective mood-elevators than the corresponding secondary amines desipramine and protriptyline the latter are considered to be more potent psychomotor activators This suggests that the mood elevating action is more related to the drug's lipophilic properties than the psychomotor activating» action.

4. Comparison of the release mechanisms

d Tubocurarine and chlorpromazine, both monomers induced Hi release on their first contact with the tissues and can therefore be included in the group which has been referred to earlier as histamine liberators However the results of the biochemical and morphological studies showed that the release mechanisms induced by these drugs differed. d Tubocurarine like compound 48/80 initiated a Ca^{++} stimulated, energy requiring process, which resulted in the release of basophil granules from the cells. Chlorpromazine — on the other hand — changed the permeability of the cell membrane, increased the cellular content of Na^{+} and probably liberated the amines with the granules still *in situ* when the granule binding sites were exposed to cation.

The common factor in the amine releasing mechanisms induced by treatment with these compounds is that granules are exposed to cations in-

tra and/or extracellularly and that granule release from the cells is not a prerequisite for amine release from the granular binding sites. Activation of a membrane-bound enzyme has been proposed as a step in the amine release induced by compound 48/80 (Högberg and Uvnäs 1957) Electron microscopic investigations (Röhlich *et al* 1971) on compound 48/80-treated mast cells have shown that very localized changes occur in the cell membrane where membrane permeability has been suggested to be changed. By contrast, chlorpromazine produces more generalized permeability changes in the membranes (III)

At the molecular level the reasons for the permeability changes are unknown and thus it is impossible to establish whether or not the same or different membrane constituents are influenced by the various releasers. However phenothiazine derivatives (Mota and Dias da Silva 1960 Zeppa and Hemmingway 1963) in concentrations below those which cause Hi release inhibit compound 48/80-induced release. This inhibition may be a consequence of the phenothiazine derivative s general permeability reducing properties (see part IV 4) In higher concentrations (> 10^{-4} M) phenothiazine derivatives potentiate the Hi releasing effect of compound 48/80 (Zeppa and Hemmingway 1963)

IV Uptake studies

1 Uptake studies on mast cells

Investigations have shown that mouse (Day and Green 1962) and rat mast cells take up 5 HT DA, NA, and Hi *in vitro* and *in vivo* (Furano and Green 1964, Cabut and Haegermark 1966 Jansson 1970, Enerbäck and Häggendal 1970 Heisler in press).

In study V the amine uptake mechanisms were investigated and the following observations made:

a All the amines except Hi were taken up by energy requiring processes and the increase in the rate of uptake decreased with increasing extra cellular amine concentrations. The latter relationship was less marked in the case of NA uptake (Fig 1) The rate of uptake for Hi increased linearly with increasing amine concentrations and did not reach a saturation level ($<6\ 10^{-4}$ M) These results are in good agreement with those previously obtained by other workers.

b The presence of Ca (1 mM) in the incubation medium significantly stimulated the rate of uptake of 5 HT and DA whereas there was no such stimulation in the case of NA and Hi

c. The amine uptake rates appeared to be almost unaffected by variations (pH 6—7 5) in the pH of the incubation medium but a significant reduction in the rate of uptake of DA was observed at pH 7 5

d. The energy-dependent amine uptake (5 HT DA, NA) appeared to follow Michaelis-Menten kinetics The different uptake rates reached the same value but the calculated affinity to the uptake mechanism was 60 times as high for 5 HT as for NA (5 HT > DA > NA)

These results could imply that energy-dependent transport with a carrier step is involved in the uptake of 5 HT DA and NA by mast cells. This is

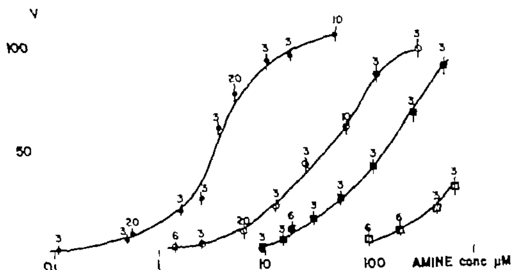


Fig. *Mast cell amine uptake as a function of extracellular amine concentration. Ordinate V uptake rate in pmol/min. Abscissa Amine concentration in μM Symbols indicate* ●—● *5 HT* O—O *DA* ■—■ *NA* □—□ *Hi.*

supported by investigations which have shown that there is a competition between 5 HT DA and NA for uptake (Heisler in press) The fact that both Ca^{++} and tricyclic psychoactive drugs influence the rates of uptake of the different amines to varying extents (see part IV 3) could indicate that separate carrier systems are involved in the transport of the amines. However this seems unlikely since these amines can compete for the neuronal uptake mechanisms in brain tissue *in vitro* (Snyder 1970 Shaskan and Snyder 1970) and for the uptake mechanism in thrombocytes (Abrams and Solomon 1969 Boullin and O'Brien 1970) whilst tricyclic psychoactive drugs retain their selective amine blocking effects. Passive diffusion of amines occurs (Green 1966) when amine concentrations higher than those which saturate amine transport are used (V)

Hi uptake is considered to be passive (Green 1966 Cabut and Haegermark 1966) but the observations that a competition for uptake between Hi and the other monoamines occured (Heisler in press) and that Hi uptake was reduced by tricyclic psychoactive drugs (part IV 3) may indicate that passive diffusion alone does not suffice to explain the uptake of Hi

Active transport of amines is considered to be coupled to a simultaneous transport of Na^+ and the rate of amine transport is dependent on the Na^+ concentration (Bogdanski and Brodie 1969) The Na^+ gradient across the

cell membrane is maintained in many cell systems by hydrolysis of ATP by a membrane-bound Na^+ K^+ activated ATP ase (Skou 1965). The uptake of amines into mast cells has also been shown to decrease in Na^+ free media (Hesler in press) Furthermore, intact mast cells can hydrolyse ATP which suggests that a membrane-bound ATP ase is present (Diamant and Krüger 1967) The transport of amines into mast cells was reduced in the presence of ouabain (10^{-4}—10^{-3} M) and N-ethylmaleimide (10^{-4}—10^{-3} M) (Hesler in press V) two substances which have been shown to inhibit membrane-bound Na^+—K^+ activated ATP ase (Skou 1965) This suggests that a similar ATP-ase may be coupled to the amine transport in mast cells. However it should be pointed out that other enzyme and metabolic inhibitors (ninhydrin, 2,4-DNP KCN) can also reduce amine uptake which implies that ATP is not the only source of energy for the transport of amines. Divalent cations have been reported to influence the activity of the membrane-bound ATP ase systems (Skou 1965), including that in mast cells (Diamant and Krüger 1967) Ca^{++} (1—2 mM) but not Mg^{++} (1—4 mM) produced a significant stimulation of the rate of transport of 5 HT and DA into mast cells (V) This could indicate that a membrane-bound ATP-ase is influenced by Ca^+ although it has been demonstrated that Ca^{++} can affect the Na^+-gradient in tissues even after inhibition of the ATP-ase system (Baker 1970)

Mast cell granules have an amine binding capacity of about 1000 mμeq/mg granules *in vitro* The endogenous amine content in mast cells which is considered to be granule-bound, is only 500 mμeq/mg granules (Uvnäs *et al* 1970) This implies that only about 50 % of the granular amine-binding capacity is utilised The unused amine-binding capacity of the granules should therefore be able to attract the different amines to the same extent, since the storage of amines appears to be mainly dependent on the ionised amino group (Bergendorff and Uvnäs, to be published) However the affinities of the amines to the granular binding sites have been shown to be different (Hi>5 HT>DA, NA, A) (Bergendorff and Uvnas, to be published) This, together with the selectivity of the amino transport mechanism could well be part of the explanation of the fact that only Hi and 5 HT have been demonstrated in rat mast cells *in vivo*

2. Influence of d-tubocurarine

d Tubocurarine ($2 \cdot 10^{-4}$ $4 \cdot 10^{-4}$ M) can block the uptake of amines (carbamoylcholine and decamethonium) in brain and muscle tissue *in vitro* (Creese and Taylor 1967 Holm 1970)

d Tubocurarine (2 10⁻⁶ 5 10⁻⁴ M) and compound 48/80 (0.001—0.05 μg/ml) were found to have no effect on amine uptake in mast cells (unpublished)

3 Influence of chlorpromazine and certain structurally-related drugs

Tricyclic neuroleptics and antidepressants block the uptake of biogenic amines in central and peripheral nerve tissue *in vitro* (Axelrod *et al.* 1962, Iversen 1967 Carlsson 1970) and also in non-neuronal tissue, such as thrombocytes (5 HT DA NA) (Stacey 1961 Abrams and Solomon 1969) and mast cells (5 HT Hi) (Green 1966)

Chlorpromazine's influence on amine uptake in mast cells was investigated (V) and the following observations made

a Chlorpromazine in concentrations of 10^{-7}—10^{-4} M reversibly inhibited the uptake of all amines studied

b The inhibition varied, 5 HT DA and NA uptake was inhibited competitively and up to 100 %, whereas the uptake of Hi was reduced by maximally about 50 %

c. The presence of Ca^{++} (2—4 mM) in the incubation medium counteracted both the uptake inhibiting effect and the uptake of ^{35}S-chlorpromazine.

In study VI the influence of the tricyclic psychoactive drugs listed in Table II on amine uptake was investigated. The uptake of Hi was reduced by neuroleptics and antidepressants. The transport of 5 HT and NA was inhibited primarily by the antidepressants. Tertiary amines were more active than secondary amines as regards 5 HT uptake, whereas the reverse was the case for NA uptake. Neuroleptics had as a rule a weak effect on 5 HT uptake, while NA uptake was relatively sensitive to this group of drugs. The inhibition appeared to be competitive in all cases DA uptake was inhibited by neuroleptics, especially the thioxanthene derivatives the inhibition was competitive, in contrast to that after treatment with antidepressants.

These results (V VI) are in accordance with the findings obtained in *in vitro* studies of the effects of these drugs on NA, DA and 5 HT uptake in brain tissue (Snyder 1970) and on 5 HT uptake in thrombocytes (Stacey

1961 Todrick and Tait 1969) A tendency towards a selective blockade of amine uptake has also been demonstrated in *in vivo* experiments (animal studies) with the drugs in question This is considered to be the explanation of the difference in therapeutic effects found on treatment with neuroleptics and antidepressants Thus neuroleptics are thought to influence primarily NA and DA turnover (van Rossum 1966 Andén *et al* 1970 Nybäck and Sedvall 1970) and antidepressants NA and 5 HT turnover (Carlsson 1970). The present results (V VI), together with the above observations, indicate that the described influence of the tricyclic psychoactive drugs is not limited to nerve tissue but also applies to other tissues which are able to take up and store biogenic amines.

4. Mechanism of uptake inhibition

Tricyclic psychoactive drugs (10^{-8}—10^{-5} M) inhibited active and passive transport in the same *in vitro* system (V VI) the active transport was inhibited by lower concentrations of the drugs. The hypotonic lysis of mast cells was also counteracted by similar concentrations of chlorpromazine (10^{-7} 5 10^{-4} M) (III) It is known from other *in vitro* systems that these drugs — in the concentrations used — can inhibit both the transport processes (Seeman 1966) and counteract the hypotonic lysis of erythrocytes. At higher concentrations ($>10^{-4}$ M) amine release and cell lysis are produced (see references part III 3)

Chlorpromazine-induced amine release from mast cells (see part III. 4) has been shown to be connected with an increase in the Na^+ permeability of the membrane and it is likely that a similar increase in permeability is the basis of the amine releasing effect of the other tricyclic psychoactive drugs investigated.

The cellular content of Na^+ in erythrocytes (Kwant and van Steveninck 1968) and in frog bladder epithelium (Mamelak *et al* 1970) is also increased by chlorpromazine in concentrations $>10^{-4}$ M These findings agree with the result in study III.

Kwant and Seeman (1969) postulated that the permeability changes observed in erythrocytes exposed to chlorpromazine are a result of both a specific binding of the drug to the membrane and an unspecific binding which takes place when the former is saturated The unspecific binding is thought to cause the increase in permeability In erythrocytes and also in other *in vitro* systems (Balzer *et al* 1968 Suarez Kurz *et al* 1970) chlorpromazine and its analogues have been shown to displace membrane-bound

V Concluding remarks

Mobilisation of cell-bound amines is considered to be the cause of certain of the adverse reactions which occur *in vivo* during treatment with drugs. However it is not known which drugs have the capacity to affect the amine-containing cells directly and which merely act as haptens in an immunological reaction (Goldstein *et al* 1969)

In concentrations corresponding to those used and found in blood during clinical treatment (Paton 1959 Waser and Harbeck 1959 Seeman and Bialy 1963) both d tubocurarine and chlorpromazine affect the amine content of mast cells (I—VI) This suggests that these drugs could also release cell-bound amines *in vivo* by a direct action and thus need not act as haptens. The adverse reactions which have been reported to occur during treatment with d tubocurarine (bronchospasm, anaphylactic shock) and chlorpromazine (dermatological symptoms) also occur on the first exposure to these drugs (for references see Introduction)

VI Summary

The mechanism of action and the structure-activity relationships (SAR) for d-tubocurarine's and chlorpromazine's Hi releasing effects and influences on the uptake of biogenic amines (5 HT DA NA, Hi) were studied *in vitro* on rat mast cells and *in situ* on perfused cat paws. The following results were obtained

1 d Tubocurarine ($>10^{-4}$ M) releases Hi by an energy-dependent, Ca^{++} stimulated process during which granules leave the cell SAR studies indicate that the presence of hydroxyl groups and the structure between the nitrogen groups in the molecule are of importance for the Hi releasing activity No connection between the Hi releasing activity of these drugs and their neuromuscular blocking effects could be demonstrated.

2 Chlorpromazine ($>10^{-5}$ M) releases Hi by a process which induces changes in the permeability of the mast cell membrane which result in an increase in the cellular Na^+ content and a reduction in the K^+ content. Tetrodotoxin reduces both the Hi release and the increase in Na^+ content. The release of Hi takes place probably in exchange for Na at the binding sites with the granules still *in situ* Ca^{++} (2—4 mM) counteract the release induced by low concentrations of chlorpromazine. SAR studies suggest that the presence of halogen groups in the tricyclic ring system and piperidine or piperazine rings in the side chain increase the activity Compounds with dimethylated side chains are more active than their monomethylated homologues. There is a significant correlation between the Hi releasing potency and the lipophilic nature of the drugs.

3 No qualitative differences in the release response could be demonstrated in the species studied.

4. 5 HT DA and NA are taken up into mast cells by energy dependent carrier-mediated transport mechanisms and Hi is taken up by diffusion. The apparent affinity for uptake is highest for 5 HT namely $3 \cdot 10^{-6}$ M: for DA the affinity is only a twentieth, and for NA a sixtieth of that for 5 HT

5 d Tubocurarine ($2 \cdot 10^{-4}$ $5 \cdot 10^{-4}$ M) does not affect amine uptake.

6 Chlorpromazine (10^{-7}—10^{-4} M) inhibits both active and passive transport. Ca^{++} (2—4 mM) counteract chlorpromazine's uptake inhibiting effect as regards 5 HT DA, NA and Hi and also the uptake of ^{35}S-chlorpromazine. These results suggest that Ca^{++} are important for chlorpromazine's mechanism of action.

7 Tricyclic neuroleptics and antidepressants inhibit active and passive transport. The uptake of 5 HT and NA is inhibited primarily by antipressants. Tertiary amines are more active than secondary amines with regard to 5 HT uptake, whilst the reverse is true for the uptake of NA. Neuroleptics have relatively little effect on 5 HT uptake and rather powerful effects on NA uptake. In each case the inhibition is competitive. DA uptake is inhibited to a greater extent by the neuroleptics, especially the thioxanthene derivatives, than by the antidepressants. The results demonstrate that these drugs also block amine uptake selectively even in tissues other than nerve tissues.

8 The investigations show that the effects of tricyclic neuroleptics and antidepressants on mast cells are the consequences of changes in permeability These can reflect the general pharmacodynamic action of the drugs. This relationship does not appear to be applicable to the neuromuscular blocking drugs.

VII Acknowledgements

My sincere thanks are due to

Professor Börje Uvnäs, who introduced me to this field of research and who has aided me in every conceivable way throughout this work

Associate Professor Sune Rosell Docents Sven Andersson Bertil Fredholm and Erik Änggård who read the manuscripts and through valuable criticism and suggestions improved them.

My co-authors Eppo v der Kleijn and Kjell Strandberg and all other members of the Department of Pharmacology who helped me in numerous ways.

Docent S A. Slorach who helped me with the English translation and fil. kand Ulf Brodin who helped me with statistical problems.

Miss Ann Katrine Thunfjord for skilful technical assistance.

The investigation has been supported by grants from Karolinska Institutet, the Swedish Medical Research Council B69-14x 39-05A, B70-14x 39-06B, B71 14x 39-07C, Svenska Sällskapet för Medicinsk Forskning Gustav V:s Forskningsfond and Åhlénstiftelsen.

the macrophage fractions of rat peritoneal cells. *Int Arch Allergy* 1967 *32* 236—245
DIAMANT B and P G KRÜGER, Histamine release from isolated rat peritoneal mast cells induced by Adenosine-5 Triphosphate. *Acta physiol. scand* 1967 *71* 291—302
DIAMANT B and C. PETERSON The effect of glucose on histamine release from isolated rat peritoneal mast cells induced by adenosine-5 triphosphate. *Acta physiol scand* 1970. *80* 299—306
DOMINO E. F., R. D HUDSON and G ZOGRAFI Substituted phenothiazines: Pharmacology and clinical structure. In: *Drugs affecting the central nervous system*. Ed: Burger A Publ Marchel Decker New York. 1968 327—397
DOUGLAS, W W., Stimulus — secretion coupling the concept and clues from chromaffin and other cells *Br J Pharmacol.* 1968 *34* 451—474.
DRAGSTEDT C. A. and E. GEBAUER FUELNEGG, Studies in anaphylaxis: I The appearance of a physiologically active substance during anaphylactic shock. *Amer J Physiol* 1932. *102* 512—519
ENERBÄCK, L. and J HÄGGENDAL, Uptake and storage of catecholamines in mucosal mast cells of the rat. *J histochem cytochem.* 1970 *18* 803—811
FURANO A. V and J P GREEN The uptake of biogenic amines by mast cells of the rat. *J Physiol.* 1964 *170* 263—271
GLASSER, H. and J KRIEGELSTEIN Die Eiweissbindung einiger Psychopharmaka mit tricyclishem Ringsystem in Abhängigkeit von ihrer chemischem Konstitution. *Naunyn-Schmiedebergs Arch Pharm.* 1970 *265* 321—334.
GOLDSTEIN A., L. ARONOW and S. M. KALMAN Immunologic basis of drug allergy In: *Principles of drug action*. 1969 476—506
GREEN J P., Binding of some biogenic amines i tissues. *Adv Pharmacol.* Academic Press, New York. 1962 349—422
GREEN J P., Synthesis, uptake and binding of histamine and 5 Hydroxytryptamine in mast cells. In *Mechanisms of release of biogenic amines* Ed Euler U S v S. Rosell and B Uvnäs. Pergamon Press. 1966 125—146
GREEN A. L., Ionization constants and water solubility of some aminoalkyl phenothiazine tranquillizers and related compounds. *J Pharm. Pharmacol* 1967 *19* 10—16
GUTH, P S. and M. A. SPRITES, Phenothiazine tranquillizers Biochemical and biophysical actions. *Int. rev neurobiol.* 1964. 7 231—278
HAINSWORTH, A. M. and W BINGHAM, An allergic circulatory collapse following administration of muscle relaxants. *Anaesth* 1970 *25* 105—109
HARRISON G A., A case of cardial arrest associated with bronchospasm and d Tubocurarine. *Aust New Zeal J Surg* 1966 *36* 40—42.
HEISLER, S In vitro studies on the uptake of biogenic amines by rat mast cells *Acta physiol scand.* In press.
HOLLISTER, L. E., Allergic reactions to tranquillizing drugs. *Ann. of Int Med* 1958 *49* 17—29
HOLM, J The effect of d Tubocurarine on the kinetics of decamethonium uptake by mouse kidney slices. *Acta pharmacol. et toxicol* 1970 *28* 370—377
HUBELL, W L., H. W McCONELL and J C. METTCALFE, Magnetic reso-

nance studies of anaesthetics in cytomembranes. *Br J Pharmacol* 1969. *35* 374—380.
HÖGBERG B and B UVNÄS Fragmentation of mast cells by some phenothiazine derivatives. *Acta chem. scand* 1957 *11* 1092.
HÖGBERG B and B UVNÄS, The mechanism of the disruption of mast cells produced by compound 48/80. *Acta physiol scand.* 1957 *41* 345—369
IVERSEN L L, *The uptake and storage of noradrenaline in sympathetic nerves* Cambridge University Press. 1967
JANSSON S-E, Effect of chlorpromazine, mepyramine, prenylamine and reserpine on 5 Hydroxytryptamine content and fine structure of rat peritoneal mast cells incubated in vitro. *Acta physiol. scand* 1970 *78* 420—430.
JANSSON S-E, Uptake of 5 Hydroxytryptamine by rat peritoneal mast cells in vitro. *Acta physiol. scand* 1970. *79* 484—492.
KAO C Y., Tetrodotoxin, saxitoxin and their significance in the study of exitation phenomena. *Pharm. Rev* 1966 *18* 997—1049.
KIELHOLZ, P and W PÖLDINGER, Die Behandlung endogener Depressionen mit Psychopharmaka. *Dt med. Wschr* 1968 *93* 701—704
KOELLE, G B., Neuromuscular blocking agents. In: *The pharmacological basis of therapeutics.* Goodman, L S and A Gilman. MacMillan Comp. New York. 1970. 601—619
KWANT W O and J van STEVENINCK, The influence of chlorpromazine on human erythrocytes. *Biochem. Pharmacol.* 1968 *17* 2215—2223
KWANT W O and P SEEMAN The displacement of membrane calcium by a local anesthetic (chlorpromazine). *Biochem. Biophys Acta.* 1969 *193* 338—349.
KWANT W O and P SEEMAN The membrane concentration of a local anesthetic (chlorpromazine) *Biochem. Biophys Acta.* 1969. *183* 530—543
LAGUNOFF D and E P BENDITT Mast cell degranulation and histamine release observed in a new in vitro system. *J exptl med* 1960. *112* 571—579
LAGUNOFF D M. T PHILLIPS O A. ISERI and E P BENDITT Isolation and preliminary characterization of rat mast cell granules. *Lab Invest* 1964 *13* 1331—1344.
LANDMARK, K. and J ØYE, The action of thioridazine and promazine on biological membranes: Relationship between ATP-ase inhibition and membrane stabilization. *Acta pharmacol et toxicol.* 1971 *29* 1—8
LANGSLET A. and M. RYG Effect of chlorpromazin and propranolol on left ventricular systolic pressure, ECG and K+ efflux in the isolated perfused rat heart. *Acta pharmacol et toxicol.* 1971 *29* 533—541
LE BLANC, J., Effect of chlorpromazine on number of mast cells and certain formed elements of blood. *Proc Soc exp Biol Med (N Y).* 1958 *97* 238
LINGJAERDE, O *Psykofarmaka. Den medikamentelle behandling av psykiske lidelser* Johan Grundt Tanum forlag, Oslo. 1970.
MAHJU M. A. and R. P MAICHEL, Accumulation of phenothiazine tranquillizers in rat brain and plasma after repeated dosage. *Biochem. Pharmacol.* 1969 *18* 2701—2710
MAMELAK, M., M. WEISSBLUTH and R. MAFFLY Effect of chlorpromazine on permeability of the toad bladder *Biochem. Pharmacol* 1970 *19* 2303—2315
MAY B., J MENKENS and E WESTERMAN Differential release of serotonin

and histamine from blood platelets of the rabbit by aliphatic and aromatic amines. *Life Sci.* 1967 *6* 2079—2085
MEYER, L., Side effects of drugs. *Excerpta Med Found* 1966 *5* 1—16 36—84.
MONGAR, J L. and H. O SCHILD Cellular mechanisms in anaphylaxis. *Physiol Rev* 1962. *42* 226—270
MORAN N C., B UVNÄS and B WESTERHOLM, Release of 5 Hydroxy tryptamine and histamine from rat mast cells. *Acta physiol. scand* 1962. *56* 26—41
MOTA, J and W DIAS da SILVA, The antianaphylactic and histamine releasing properties of the antihistamines. Their effect on the mast cells. *Br J Pharmacol.* 1960 *15* 396—412.
MOTA, J The behavior of mast cells in anaphylaxis. *Intern Rev Cytol.* 1963 *15* 363—397
NYBÄCK, H. and G SEDVALL, Further studies on the accumulation and disappearance of catecholamines formed from Tyrosine-14C in mouse brain. Effect of some phenothiazine analogues. *European J Pharmacol.* 1970. *10* 193—205
PAASONEN M. K., On the mechanisms of action of chlorpromazine in relea sing 5 Hydroxytryptamine from blood platelets in vitro. *Naunyn Schmiedebergs Arch Pharm* 1964. *248* 223—240.
PADAVER, I and A. S GORDON Isolation of mast cells from other cellular elements of the rat peritoneal fluid. *Proc Soc exp Biol Med* 1955 *88* 29—31
PALM, D H., H. GROBECKER and J J BAK, Membrane effects of catecholamine drugs. In: *New aspects of storage and release of catecholamines* Ed: Schümann, H. L and G Kroneberg. Springer Verlag. 1970. 488—498
PATON W D M., Histamine release by compounds of simple chemical structure *Pharmacol. Rev* 1957 *9* 269—328
PATON W D M., The effects of muscle relaxants other than muscular relaxation. *Anesth* 1959 *20* 453—463
PFEIFFER, C. C., V ILIEV L. GOLDSTEIN and E. H. JENNEY Serum polyamine levels in schizophrenics and other objective criteria of clinical status. In: *Schizophrenia current concepts and research* Ed Siva Sankar D V (N Y) 1969 557—563
POPOVA, A. D., S. A. SLORACH and B UVNÄS, The binding of toluidine blue and chlorpromazine to isolated rat mast cell granules. *Acta pharmacol et toxicol* 1971 *30* 215—224
RILEY J F The effects of histamine-liberators on the mast cells of the rat. *J Path Bact* 1953 *65* 471—479
RILEY J F *The mast cells* E & S Livingstone Ltd. Edinburgh & London. 1959
RILEY J F and G B. WEST The occurence of histamine in mast cells In *Handbuch der exptl. Pharmacol.* Ed: Rocha E Silva, M. Springer Verlag. Berlin. 1966 *181* 116—135
ROBINSON J D J LOWINGER and B BETTINGER, Chlorpromazine: differential effects on membrane-bound enzymes from rat brain. *Biochem Pharmacol* 1968 *17* 1113—1116
ROCHA E SILVA M. and H. O SCHILD The release of histamine by d Tubocurarine from the isolated diaphragm of the rat. *J Physiol. (Lond)* 1949 *109* 448—458

ROSSUM, J M van The significance of dopamine receptor blockade for the mechanism of action of neuroleptic drugs *Arch Int Pharmacol* 1966 *160*. 492—494.

ROTHSCHILD A. M., Histamine release by basi compounds IN: *Handbuch der exptl. Pharmacol* Ed: Rocha E Silva, M Springer Verlag Berl n. 1966 *18I* 386—438

RÖHLICH, P., P ANDERSSON and B UVNÄS Electron microscopic observations on compound 48/80 induced degranulation in rat mast cells. Evidence for sequential exocytosis of storage granules. *J Cell Biol* 1971 *51* 465—483

SALEM, M. R., Y KIM and A. A. ELETR, Histamine release following intravenous injection of d Tubocurarine *Anesth* 1968 *29* 380—382

SCHMID H Chemie des Calebassencurare. *Bull Schweiz Akad med Wiss* 1967 *22* 415—428

SCHWARTZ, K. J Experimental untersuchen zur photoallergie gegen sulfanilamid und chlorpromazine. *Dermatologia.* 1969 139 Suppl 5

SEEMAN P M., Membrane stabilization of drugs. *Int Rev Neurobiol* 1966 *9* 145—221

SEEMAN P M. and H S BIALY The surface activity of Tranquilizers *Biochem. Pharmacol* 1963 *12* 1181—1191

SELYE, H., *The mast cells* Butterworths, Washington 1965

SHASKAN E. G and S H SNYDER, Kinetics of sereton n accumulation into slices from rat brain: Relationship to catecholamine uptake. *J Pharmacol exp Ther* 1970 *175* 404—418

SHORE, P A., A. BURKHALTER and V H COHN Jr A method for the fluorimetric assay of histamine in tissues. *J Pharmacol. exptl Ther* 1959 *127* 182—186

SKOU J C., Enzymatic basis for active transport of Na and K+ across cell membrane. *Physiol Rev* 1965 *45* 596—617

SLORACH, S and B. UVNÄS Amine formation by rat mast cells in vitro. *Acta physiol. scand* 1968 *73* 457—470.

SMITH D E. and Y S LEWIS, Electron microscopy of the tissue mast cells. *J biophys biochem. cytol* 1957 *3* 9—14

SMITH, D E., The nature of the secretory activity of the mast cell. *Am. J of Physiol.* 1958 *193* 573—575

SNYDER, S H., Putative neurotransmitters in the brain Selective neuronal uptake, subcellular localization and interactions with centrally acting drugs. *Biol. Psych* 1970. *2* 367—389

STACEY R. S., Uptake of 5 Hydroxytryptamine by platelets. *Br J Pharmacol.* 1961 *16* 284—295

STRANDBERG K., Ca++ dependence of histamine release and formation of slow reacting substance in the cat paw *Acta physiol. scand.* 1971 *82* 500—508

STRANDBERG, K., Release of histamine and formation of slow reacting substance in the cat paw perfused with compound 48/80. *Acta physiol. scand* 1971 *82* 47—58

SUAREZ-KURTZ, G C. P BIANCHI and P KRUPP Effect of local anesthetics on radiocalcium binding in nerve. *European J Pharmacol.* 1970 *10* 91—100.

TAKKI, S. and T TANNISTO Severe bronchospasm and circulatory collapse

following the administration of d Tubocurarine. *Ann. of clin. research* 1971 *3* 112—115
THON I L. and B UVNÄS Mode of storage of histamine in mast cells. *Acta physiol. scand* 1966 *67* 455—470
THON I. L., and B. UVNÄS Degranulation and histamine release two consecutive steps in the response of rat mast cells to compound 48/80. *Acta physiol scand* 1967 *71* 303—315
TODRICK, A. and A. C. TAIT The inhibition of human platelet 5 Hydroxytryptamine uptake by tricyclic antidepressive drugs. The relation between structure and potency *J Pharm. Pharmacol* 1969 *21* 751—769
TUOMISTO J., Release of histamine from rabbit blood platelets in vitro. *Ann. Med exp Fenn.* 1968 *46* 318—330
UVNÄS, B., Metabolic and non-metabolic processes in the mechanism of histamine release from mast cells. Int *Biochemistry of the allergic reactions* Ed: Austen, K. F and E. L. Becker Blackwell Scientific public, Oxford. 1968 131—140.
UVNÄS, B and I. L. THON Isolation of biological intact mast cells. *Exp Cell. Res* 1959 *18* 512—520.
UVNÄS, B and I. L. THON A physico-chemical model of histamine release from mast cells. Proc. Int. Wenner-Gren Center Symp., Stockholm 1965 In: *Mechanism of release of biogenic amines* Ed: Euler U S. v S. Rosell and B Uvnäs. Pergamon Press. Oxford. 1966 361—371
UVNÄS B C. H. ÅBORG and A. BERGENDORFF Storage of histamine in mast cells. Evidence for an ionic binding of histamine to protein carboxyls in the granule heparin protein complex. *Acta physiol scand* 1970. *suppl. 336* 1—26
VAPAATALO H. I., Studies in the mechanisms of chlorpromazine induced adrenaline release from bovine adrenal gland. *Ann Med exp Fenn* 1968 *46* 345—352
VAPAATALO H. I L. ATHEE and M. K. PAASONEN Release of adrenaline and noradrenaline by some phenothiazines from the bovine adrenal gland. *Ann Med exp Fenn.* 1966 *44* 464—468
WASER, P G and P HARBECK, Erste klinische Anwendung der Calebassenalkaloide Toxiferin I and Curarin I *Der Anaesth* 1959 *8* 193—198
WASER P G., Die pharmacologie der Calebassenalkaloide. *Bull. Schweiz Acad med Wiss* 1966 *22* 486—500.
WESTERHOLM, B., *Frisättning av histamin och 5 Hydroxytryptamin in vitro En djurexperimentell serie* Thesis, Stockholm. 1964.
WESTGATE, H. D and F H. van BERGEN Changes in histamine blood levels following d Tubocurarine administration. *Canad Anaesth Soc J* 1962 *9* 497—503
ZEPPA R. and G C. HEMMINGWAY Inhibition of histamine release from mast cells. *Surgical Forum.* 1963 *14* 56—57
ZIMMERMAN H. J and J KENDLER, Relationship between structure of Phenothiazines and *in vitro* cytotoxicity *Proc Soc of exp Biol. Med* 1970 *135* 201—205

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 377

From the Department of Medical Pharmacology
Uppsala University Sweden

DOUBLE ISOTOPE DILUTION DERIVATIVE TECHNIQUE FOR MEASUREMENT OF CATECHOLAMINES

By

LEIF RENTZHOG

UPPSALA 1972

ERRATA

Acta physiol scand., 1972 Suppl 377

Page 39 line 1 as triacetate after the acetylation should read *initially*

Page 45 3rd paragraph line 7 to 1/2 or 1/3 of the original ratio should read 'to *2/3* or 1/3 of the original ratio

Page 47 48 52 figure-text fig 4 5 8 ^{3}H-activity (black bars) and ^{14}C-activity (white bars) should read ^{3}H-activity (*white bars*) and ^{14}C-activity (*black bars*)

Page 56 table XIII the figures in the first column (Run and fig) should read (from top to bottom) '201/*4* 202/*6* 203/*6* 204/*6* *303*/*7* 302/*7* *301*/*7* 401/*8*

Page 65 4th paragraph line 6 Paasonen and Dews 1958 used *bioassay* not fluorometric technique

Page 69 2nd paragraph, line 20 'and 0 4 ng(I) and 0 ng (II) apparent noradrenaline should read 'and *0 8* ng(I) and 0 ng (II) apparent noradrenaline

Page 81 fig 10 2nd line under the x-axis 5 50 100 200 should read '5 *50 100 500*'

Page 90 2nd paragraph line 2 3 'In order to improve the catecholamine determinations have also been made during the 1960's Gas chromatographic separation of non-derivated catecholamines should read 'In order to im prove the determinations of *catecholamines and related compounds* have also been made during the 1960's. Gas chromatographic separation of non derivated *phenolic amines*

Page 90 2nd paragraph line 13–14 'acetylation of hydroxyl groups and silylation of the amino group should read acetylation of *amino* and *phenolic hydroxyl* groups and *silylation of the alcoholic hydroxyl* group

DOUBLE ISOTOPE DILUTION DERIVATIVE TECHNIQUE FOR MEASUREMENT OF CATECHOLAMINES

Akademisk avhandling

som med vederbörligt tillstånd av Medicinska Fakulteten vid Uppsala Universitet för vinnande av medicine doktorsgrad framlägges till offentlig granskning å Institutionen för Farmakologi och Toxikologi, föreläsningssal B 21 Uppsala Universitets Biomedicinska Centrum torsdagen den 4 maj 1972 kl. 9 15 (precis)

av

Leif Rentzhog
med lic., Norrl.

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 377

Kå-We Tryck Uppsala 1972

From the Department of Medical Pharmacology
Uppsala University Sweden

DOUBLE ISOTOPE DILUTION DERIVATIVE TECHNIQUE FOR MEASUREMENT OF CATECHOLAMINES

By

LEIF RENTZHOG

UPPSALA 1972

To my father

Contents

In the last twenty years research on catecholamines has expanded rapidly One of the cornerstones has been the methods allowing quantitative determination of relatively small amounts of catecholamines by oxidation to fluorescent trihydroxindole (THI) derivatives introduced by Ehrlen in 1948 and Lund in 1949 and the ethylenediamine condensation method developed by Weil Malherbe and Bone 195? The sensitivity of the fluorometric method has been continuously improved by several modifications of the original methods, see reviews from the first and second Symposium on Catecholamines (Bethesda 1958 and Milan 1965) One remaining problem however is the difficulty of measuring several catecholamines and their metabolites in the same sample when there are large differences in concentration between them without an extensive primary separation procedure involving a considerable risk of destruction of these easily oxidizable substances (Häggendal 1966 Iversen 1967) The presence of other substances with fluorescence spectra similar to those of the catecholamines can also complicate determinations in biological material (Carruthers *et al.* 1970)

In the mid 1960 s we began to be interested in the importance of minority catecholamines in several tissues. These are difficult to measure in the presence of a large excess of noradrenaline The question whether adrenaline occurs in the mammalian brain for instance has been one subject of discussion (Gunne 1962) Another interesting question discussed from time to time is whether small amounts of other catecholamines than those reported may be of some physiological importance in the body Lockett's study 1954 with chromatographic isolation of an isoprenaline-like substance from the adrenals is a case in point

To make further investigations possible in this field our aim was to find a quantitative determination method where several different catecholamines can be measured with minimal interference from each other This should be possible with a double isotope technique where each catecholamine in a sample can be submitted to extensive individual purification To permit this, sufficiently stable catecholamine derivatives must be prepared Bretschneider (1946) and Welsh (1952), using two different techniques, originally described the preparation of catecholamine acetyl derivatives Goldstein Friedhoff and Simmonds (1959) showed that noradrenaline and adrenaline triacetate were stable enough to permit paper chromatographic separation. The acetylation procedure has been used with great success for quantitative assay of several other substances by double isotope dilution techniques (see review by

Whitehead and Dean 1968) ever since its initial introduction for estimation of aldosterone by Kliman and Peterson (1960) Therefore the acetyl derivatives seemed promising for further studies.

We have found that acetylation of several catecholamines and their metabolites can take place with good yield on a microscale both with the technique of Welsh and with that of Bretschneider The present investigation shows the possibility of quantitative assay of adrenaline and noradrenaline by double isotope procedures in which tracers of ^{14}C-labelled (or ^{3}H-labelled) catecholamines are added and acetylation with acetic ^{3}H anhydride (or acetic ^{14}C anhydride) is performed After chromatographic separation of the acetyl derivatives formed the amount of unknown catecholamine can be calculated from the $^{3}H/^{14}C$ ratio obtained Owing to the addition of the tracer catecholamines no correction for losses during the acetylation and separation steps is necessary and only the relative recovery of ^{3}H compared with ^{14}C is of importance for the results. However several problems arise in determinations by these methods.

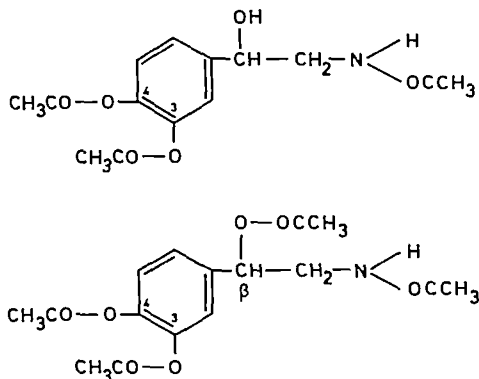


Fig. 1 3 4,N-triacetyl noradrenaline (above) and 3,4,β,N-tetraacetyl noradrenaline

In the following the different steps in the acetylation method for determination of catecholamines by acetylation double isotope techniques will be discussed The first part will describe and discuss our method using partial acetylation according to the procedure described by Welsh (1957) This technique gives the 3 4,N-triacetyl derivatives of adrenaline and noradrenaline (fig. 1) The second part describes and discusses the technique using the acetylation method of Bretschneider (1946) which gives the 3 4 β,N-tetraacetyl derivatives of adrenaline and noradrenaline (fig. 1) An extensive comparative discussion and a review of alternative methods forms the third part The main features of the triacetylation method have been presented briefly in an earlier report (Rentzhog 1970)

PART 1

DOUBLE ISOTOPE DILUTION TECHNIQUE WITH PARTIAL ACETYLATION OF CATECHOLAMINES (PREPARATION OF TRIACETATE)

Chapter I

MATERIALS and METHODS

The individual steps of the analytical procedure are

A. Preparation of extract
B. Adding of radioactive tracers.
C. Isolation on alumina.
D Concentrating the eluate
E. Acetylation
F Adding the carriers.
G Extraction of catecholamine triacetates.
H. Separation of triacetates by chromatography
I Purification by repeated chromatography
J Radioactive measurements.
K. Standards.
L. Calculations.

A. Preparation of extract

Materials

Perchloric acid Suprapur® Merck Darmstadt Germany

EDTA disodium salt Titriplex III® A.R. Merck.

Tri-R tissue homogenizer with teflon pestle Tri R Rockville Centre N Y

Polytron® Kinematica, Luzern Switzerland (for homogenization of harder tissue)

Sorvall® RC 2 Refrigerated Superspeed Centrifuge Ivan Sorvall Inc Norwalk, Conn.

Procedure

Up to 2 g of tissue is homogenized carefully in 10 ml 0 4 N perchloric acid containing 1% EDTA in an ice-bath The homogenate is transferred to a 20 ml polyethylene centrifuge tube and is allowed to stand in an ice-bath for 30 min After extraction the homogenate is centrifuged in a refrigerated centrifuge (0°C) for 10 min at about 9000 g. The supernatant is carefully decanted into a new centrifuge tube The precipitate is reextracted with 5 ml 0 4 N perchloric acid The supernatants are pooled

B Adding of radioactive tracers

Materials

DL-Adrenaline 7 ^{14}C and DL-Noradrenaline 7 ^{14}C specific activity 40-60 Ci/mol These substances were purchased in 0 1 N acetic acid solution from New England Nuclear Corporation Frankfurt/Main Germany

n Butanol A.R. Merck.

Acetic acid glacial, A.R., Merck.

Ethyl methyl ketone A.R. Merck

Propionic acid A.R. Erba Milano Italy

TLC-plates Cellulose F pre-coated Merck

Silica gel HF_{254} acc to Stahl Merck (Preparation of plates see below)

Purity control

The stock solutions are kept at −20°C The radiochemical purity of the catecholamine tracers is checked monthly by thin-layer chromatography in the following systems

n-butanol acetic acid water (12:3:5) on TLC cellulose and TLC silica gel

Ethyl methyl ketone propionic acid water (15:5:6) on TLC cellulose

The chromatograms are scanned by scraping fractions into scintillation fluid and counting in a Packard Tri-Carb liquid scintillation spectrometer Batches with a radiochemical purity less than 90% are repurified by passage over alumina

Procedure

^{14}C-labelled catecholamines are added as tracers for the substances that are to be determined The ^{14}C-tracers added to the samples and the tracers added to the standards (see moment K) are taken at the same time from the same stock solutions. The proportion between the volumes of ^{14}C-catecholamines added to the sample and to the standard I (see K) must be accurately known If one expects that the unknown amounts of each catecholamine in the sample are

>0.1 μg, $2 \cdot 10^4$ cpm of each tracer is used. If the unknown amounts of catecholamine in the sample can not be predicted or if the expected amounts are ≤ 0.1 μg the quantities of the tracers must be reduced. The chemical amounts of tracer should not be much higher than the expected amounts of unknown catecholamine in the samples. However, the amounts of tracer added should not be less than $3 \cdot 10^3$ cpm in order to give enough ^{14}C-counts in the final sample.

C. Isolation on alumina

Materials

Titriplex III® A.R. Merck

Potassium carbonate A.R. Merck.

Alumina neutral grade I Woelm Eschwege Germany

Sodium hydroxide Titrisol® Merck

Acetone A.R., Merck.

Ethanol spectrophotometric grade V o S Sweden

Formic acid A.R., Merck.

pH-meter Titrator TTT 1b Radiometer Copenhagen Denmark

Microid flask shaker Griffin and Tatlock Ltd London England

Preparation of alumina

The aluminium oxide is prepared according to Anton and Sayre (1962). 100 g alumina is added to 500 ml 2 N HCl and heated to 100 C for 45 min under continuous stirring. The alumina is then washed twice with fresh 2 N HCl 250 ml at 70°C for 10 min and finally with 500 ml 2 N HCl at 50 C for 10 min. It is then washed several times with water until pH 3 3.5 is reached. Each time the supernatant fluid is discarded together with the suspended aluminium oxide particles. Finally the alumina is activated at 120°C for one hour and at 200 C for two hours.

Procedure

The pH of the extract is adjusted to about 4 with 5 N potassium carbonate. The potassium perchlorate is spun down at 0°C. The clear supernatant is then transferred to a 50 ml round bottom flask and 400 mg alumina and 200 mg EDTA are added. Under careful constant manual agitation of the flask the pH is adjusted with 2 N NaOH and fine-adjusted with 0.2 N NaOH to pH 8.3. The agitation and stirring are continued for 5 min at this pH. The alumina is then allowed to settle and the supernatant is aspirated off and discarded. The

sedimented alumina is transferred quantitatively with bidistilled water into a 10 ml glass tube with a polyethylene cap The alumina is then washed by shaking 3 x 2 min with 10 ml bidistilled water and once for 2 min with each of 10 ml ethanol and 10 ml acetone The catecholamines are then eluted by shaking the alumina for 1 2 min with 2 ml 0 1 N formic acid and for further 1 min with 1 ml. The pooled eluates are transferred to a 10 ml glass centrifuge tube and centrifuged at 20 000 g for 15 min to remove small residues of alumina.

D Concentrating the eluate
Materials
Freeze dryer HBS B 65 New Brunswick Scientific New Brunswick NJ

Procedure
The sample is transferred into a 10 ml pear-shaped glass flask and freeze-dried This process is allowed to proceed overnight The lyophilized sample is finally dissolved by the addition of 200 μl 1 mM HCl and the flask stoppered with an ungreased glass stopper The sample can be kept in the refrigerator at least several hours

E. Acetylation
Materials
Hydrochloric acid Titrisol® Merck
Sodium hydrogen carbonate A.R., Merck.
pH indicator paper Fluka, Buchs Switzerland
Microsyringe Hamilton The Hague Holland
Rotary vacuum pump S 1 Leybold-Heraeus, Koln Germany
Acetic ^{3}H anhydride specific activity 100 Ci/mol New England Nuclear Corp

The acetic ^{3}H anhydride arrives in benzene solution (20:80 vol/vol) in ampoules with standard taper joints of 20 mCi This is sufficient for 10-12 acetylations. The acetic ^{3}H anhydride is doubly distilled on the day of shipment from NEN to remove any trace of nonvolatile labelled impurities It is stored in a refrigerator at +5°C Immediately prior to the acetylation the acetic ^{3}H anhydride is redistilled under vacuum For this a simple procedure has been developed using an N-shaped glass tube (see fig. 2) Before distillation starts the acetic anhydride is frozen in acetone/CO_2-ice During

distillation the ampoule is kept at room temperature and the lower corner of the N is immersed in acetone/CO_2-ice After the distillation the glass tube can be closed with a ground-glass stopper and used as a reservoir for the freshly distilled acetic ^{3}H anhydride which however should be used up at once

Procedure

To perform the acetylation 7-8 µl acetic ^{3}H anhydride benzene (?0:80 vol/vol) is added with a Hamilton® syringe to the HCl-solution of the sample in the glass pear Immediately afterwards about 10 mg of $NaHCO_3$ (powder) is added An excess is aimed at If all of it dissolves a little more is added The sample is shaken for 30 sec and allowed to stand for 5 min The pH is then adjusted to about 3 with 200-300 µl N HCl to neutralize the excess of $NaHCO_3$ The sample is then allowed to stand for 30 min to make sure that the excess of acetic anhydride is hydrolyzed

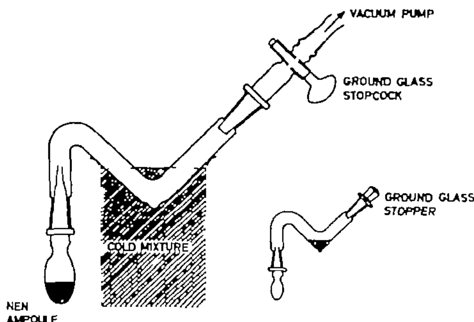


Fig. 2. Technique for redistillation of radioactive acetic anhydride.

F Adding of carriers

Materials

DL-Adrenaline bitartrate British Drug Houses Poole England

DL-Noradrenaline bitartrate Astra Södertälje Sweden

Acetic anhydride A R. Merck.

Sodium hydrogen carbonate A.R., Merck.

Chloroform A.R., Merck.

Methanol A.R. Merck

Reagents for chromatography see below

Preparation of carriers

200 mg catecholamine is dissolved in 9 ml doubly distilled water 0.3 ml acetic anhydride is added and immediately afterwards 0.6 g $NaHCO_3$ in small portions. When the evolution of carbon dioxide has ceased the mixture is extracted with 3 x 10 ml chloroform. The extract is washed with 3 x 10 ml water and then concentrated in a current of air The wet residue is freeze-dried The catecholamine triacetate is dissolved in 0.5 ml methanol and purified by chromatography in system S_1 on a preparative silica gel plate (see below) The triacetate is eluted with 2 x 5 ml methanol which is then evaporated in a current of air The purified carrier is dissolved in methanol and kept at $-20^{\circ}C$ It is possible to keep the carrier at a higher temperature for a week or so but we have found that if the carrier stands for a long time at 4-6 C or at room temperature it causes chemoluminiscence when added to scintillation fluid

Procedure

About 100 μg unlabelled triacetate of each catecholamine that is to be determined are added as carriers in methanol solution.

G Extraction of catecholamine triacetates

Materials

Chloroform A.R., Merck

Teflon® thread seal (a thin tape), Habia, Uppsala Sweden

Microid flask shaker Griffin and Tatlock Ltd

Procedure

The acetylated catecholamines are extracted from the aqueous reaction mixture by shaking for 10 min with three 2 ml portions of chloroform Before shaking, the joint between the pear and its stopper is carefully sealed with Teflon® tape to minimize the risk of contaminating the laboratory The pooled organic phase

is transferred into another 10 ml pear and evaporated to dryness in a current of air followed by lyophilization For subsequent chromatography the residue is taken up in ?00 μl methanol The solution is kept in the refrigerator

II I Separation and purification of triacetates by chromatography

Materials

Acetic acid glacial A.R., Merck
Acetone A.R., Merck.
Chloroform A.R., Merck.
Cyclohexane A.R., Merck
Ethyl acetate A R., Merck
Formamide A.R Merck
Methanol, A.R., Merck
Pyridine A R., Mallinckrodt St Louis, Mo
Toluene A.R. Fisher Fair Lawn N.J
Ethylenediamine purum Kebo Stockholm Sweden
Sodium fluorescein May and Baker Dagenham England
3-hydroxypyrene 5,8 10-trisulfonic acid sodium salt Bayer Leverkusen Germany
Silica gel HF_{254} acc to Stahl Merck.
Whatman® chromatography paper no *1 Balston Maidstone England*
Chromatography paper MN 2214 Machery Nagel and Co Dürren Germany
Desaga thin layer spreading equipment Desaga Heidelberg, Germany
UV lamp Luma, Stockholm Sweden
Microtrol microsyringe Drummond Briomall Pa.

Chromatography systems, listed in the order in which they are applied

S_1 Chloroform acetic acid (2 1) Silica gel TLC
S_2-S_3 Chloroform methanol (9 1) (S_2) Chloroform acetic acid (1 1) (S_3) Two dimensional silica gel TLC
P_4 Toluene ethyl acetate methanol water (10 15 5) (organic phase) Paper chromatography
S_4 Chloroform cyclohexane acetic acid methanol (5 3 1 1). Silica gel TLC
P Toluene ethyl acetate (3 1) (formamide-saturated) Paper chromatography
S_7 Chloroform pyridine (9 1) Silica gel TLC
Rf values are shown in table VI on page 32

Thin layer chromatography

Thin layers of silica gel 0.25 mm thick, are prepared by making a slurry of silica gel 30 g and water 90 ml and spreading this on glass plates (5 x 20 cm or 20 x 20 cm), using a Desaga equipment. The plates are allowed to dry and are activated at 110°C for 30 min They are stored in room atmosphere The sample is applied to the plate 2 cm from the base using a microsyringe with disposable bore A new bore is used for each sample to avoid contamination. The solvent front is allowed to migrate 12 cm above the spotting line using an ascending technique at room temperature without previous equilibration The plates are dried at room temperature and the acetyl derivatives are identified in UV light (254 mμ) When system S_7 is used the plates are sprayed with 3-hydroxypyrene 5,8 10-trisulfonic acid (0.2% vol/vol in water) for identification. The spots are eluted by shaking for 10 min with 2 ml methanol and for a further 10 min with 1 ml

Paper chromatography

In chromatography P_4 the paper Whatman no 1 (3.5 x 57 cm) is used without pretreatment

In chromatography P_6 the paper MN 2214 (3.5 x 57 cm) is impregnated in formamide (30% formamide vol/vol in acetone for 5 min) and allowed to dry at room temperature for 60 min before use

In both paper chromatography systems we used a descending technique at room temperature without previous equilibration The sample is applied with a microsyringe 5 cm from the base of the paper The solvent front is allowed to pass over the front edge of the paper The chromatograms are developed for 10 h (adrenaline triacetate) and 16 h (noradrenaline triacetate) in system P_4 and for 24 h (adrenaline triacetate) and 40 h (noradrenaline triacetate) in system P_6 For identification of the spots, which must be done very carefully a narrow strip in the centre of the chromatogram is cut out and sprayed with sodium fluorescein (50 mg sodium fluorescein dissolved in 100 ml 50% vol/vol methanol in water) followed by ethylenediamine water 1 1 This gives a very good contrast in UV light (254 mμ) However the test strip from the *chromatogram must be as small as possible as the acetyl derivatives on this strip* are destroyed by the ethylenediamine Elution from the chromatograms is performed by shaking with 2 + 1 ml methanol after chromatography P_4 and with 2 + 1 ml chloroform after chromatography P_6

Procedure

Separation of the different catecholamine triacetates is accomplished by thin layer chromatography in system S_1 After elution of the separate spots with

methanol the eluates are freeze-dried and dissolved in 200 µl methanol Aliquots of 5 µl are counted by liquid scintillation The catecholamine triacetates are then rechromatographed separately in S_2 S_7 After each chromatography the eluates are freeze-dried and dissolved in 200 µl methanol and aliquots taken for determination of the $^{3}H/^{14}C$ ratio

The volume of the aliquot depends on the cpm in the sample The chromatographic purification of the catecholamine derivatives is continued until constancy of the isotope ratios is attained Usually this happens before system S_7 and sometimes before P_4 These systems are then not used

J Radioactive measurements

Materials·

2,5-diphenyloxazole (PPO) scintillation grade Packard Downers Grove Ill

1 4-bis-2(4)-methyl 5-phenyl-oxazolylbenzene (dimethyl-POPOP) scintillation grade Packard

Naphtalene purum British Drug Houses Ltd

Dioxane purum Biotek, Göteborg, Sweden

Methanol A.R., Merck.

Tri-Carb liquid scintillation spectrometer modell 3010 Packard

Polyethylene vials Packard

Radioactive counting

The aliquots of the eluates (from chromatograms) are counted in a scintillation liquid consisting of 4 g PPO 50 mg dimethyl-POPOP and 125 g naphtalene to 1000 ml dioxane and 100 ml methanol Variations in voltage are checked with ^{14}C and ^{3}H standards at each count. No corrections for quenching are usually necessary but suspicious results are checked for quenching with an external standard and in certain samples with addition of known amounts of ^{3}H and ^{14}C toluene For background corrections blank samples are prepared from non-radioactive catecholamine triacetates which have been chromatographed in the different solvent systems used eluted in methanol lyophilized and dissolved in 200 µl methanol The background values obtained vary somewhat with eluates from different chromatography systems and also depend on the volume of the final methanol solution added to the scintillation mixture From the blank samples an individual background correction is made for each kind of chromatography 10 000 counts were accumulated if at all possible Samples were however not counted for longer than 50 min They always were more than 5 times background. The efficiencies were determined from ^{3}H and ^{14}C

toluene solutions (New England Nuclear Corp) Tritium was determined in the first channel at 26 4% efficiency and in the second channel at < 0.01% The efficiency for measuring ^{14}C was *8.3% and 58 1% respectively*

K. Standards

Materials

DL Adrenaline-7 ^{14}C DL-Noradrenaline 7 ^{14}C 40-60 Ci/mol. Acetic ^{3}H anhydride 100 Ci/mol New England Nuclear Corporation

DL Adrenaline bitartrate British Drug Houses.

DL Noradrenaline bitartrate Astra.

In critical experiments the unlabelled catecholamines are purified by repeated crystallization in ethanol water 10 vol 95% ethanol are added to an equal weight of catecholamine salt in bidistilled water at 50 C Crystallization takes place at −10 C after seeding The crystals are washed in 95% ethanol and ether

Procedure

Two kinds of standards are used in each analytical run which as a rule embraces 10 unknown samples and utilizes one batch of acetic anhydride Standard I contains ^{14}C-adrenaline and ^{14}C-noradrenaline (about 10^5 cpm ($\sim 0.5\ \mu g$) of each diluted from the stock solutions to 50 µl in 1 mM HCl) and accurately known amounts of unlabelled adrenaline and noradrenaline Standard II contains about the same amounts of ^{14}C-adrenaline and ^{14}C-noradrenaline as standard I but no unlabelled catecholamines.

The ^{14}C-tracers used in the standards and the tracers added to the samples (see moment C) are taken at the same time from the same stock solutions. The proportion between the volumes of the ^{14}C-catecholamine solutions added to *standard I and to the samples must be accurately known It is not necessary that* the volumes added to standard II are exactly known.

The amounts of unlabelled catecholamines added to standard I are about 5 µg (in 50 µl 1 mM HCl) of each when standard I is run together with samples which one expects to contain > 0 1 µg of each catecholamine If one expects that any of the samples contains ≤ 0 1 µg of an unknown the amount of the corresponding unlabelled catecholamine added to standard I should be reduced and depends on the expected $^{3}H/^{14}C$ ratios of the samples. In this case one aims at similar values of the $^{3}H/^{14}C$ ratios in standard I and the samples. If one for instance expects that the samples contain about 0.01 µg of one catecholamine 0.5 µg of the same catecholamine is used in standard I If the amounts of catecholamine in the samples are expected to be low but cannot be approximately predicted 2 3 standard I are run with different amounts of

unlabelled catecholamine In the calculation one then uses the $^3H/^{14}C$ ratio of standard that is closest to that of the sample The standards are acetylated with acetic ^3H anhydride with the same technique and at the same time as the samples (see moment E) They are then processed as described for the samples above (F I) until constant $^3H/^{14}C$ ratios are obtained The ratios for standard I and II are used in the calculations of the results for the whole analytical run

L. Calculations

The amount x of a certain unlabelled catecholamine in a sample in micrograms can be calculated from the formula

$$x = a \; \frac{t_s}{t_I} \; \frac{r_s - r_{II}}{r_I - r_{II}}$$

where

a = μg unlabelled catecholamine added to standard I
t_s = cpm ^{14}C tracer added to sample
t_I = cpm ^{14}C tracer added to standard I
r_s = $^3H/^{14}C$ ratio of sample
r_I = $^3H/^{14}C$ ratio of standard I
r_{II} = $^3H/^{14}C$ ratio of standard II

The isotope ratios r_s r_I and r_{II} are the mean values of the $^3H/^{14}C$ ratios in repeated chromatographies after essential constancy has been obtained

RATIONALE OF THE METHOD

In this chapter the different steps of the method are discussed and the experiments reported on which the various choices have been based.

Homogenization and protein precipitation

For protein precipitation we use 0 4 N perchloric acid When different protein precipitation methods are used with the THI fluorometric technique somewhat *varying results are obtained mainly because of differences in the blank values* (Anton and Sayre 1962) On comparison between 0 4 N perchloric acid and other protein precipitation agents such as 5% trichloroacetic acid or acidified absolute ethanol we found no difference in the absorption on alumina the degree of acetylation of the catecholamines or the background after the acetylation

Since one of the aims of this method was to allow demonstration of small quantities of isoprenaline or other minority catecholamines which may possibly be present in biological tissue it was important to exclude artifacts due to the action of perchloric acid on adrenaline or noradrenaline during the homogenization process That catecholamines can be changed in a strongly acid environment is well known (Roberts 1966 Forrest *et al.* 1970) Roberts for example has shown that if adrenaline is dissolved in hydrochloric acid and chromatographed a so-called double spot with an Rf value similar to that of isoprenaline can be obtained Several authors (Shepherd and West 1952 Beckett and Choulis 1963) have reported that the presence of traces of trichloroacetic acid may also give rise to the double spot phenomenon in chromatographies of catecholamines on paper and cellulose

In our own chromatographic studies of perchloric acid extracted *catecholamines we found that on polyamide TLC in isobutanol acetic acid* cyclohexane (80 7 10), adrenaline could give rise to a product with a similar Rf value to isoprenaline In order to find out whether this unknown spot represented a breakdown product of adrenaline EDTA and sodium bisulphite were added to solutions of adrenaline in 0 4 N perchloric acid This did not affect the double spot phenomenon When on the other hand the perchloric acid solution was neutralized with sodium hydroxide before the chromatography only one spot was seen This indicates that the double spot phenomenon seen in chromatography of perchloric acid extracted catechol

amines is probably due to the fact that the catecholamines migrate in the chromatogram partly as free base and partly in salt form an assumption which agrees with previous findings concerning trichloroacetic acid (Beckett and Choulis 1963). No destruction of the catecholamines thus appears to take place during extraction with perchloric acid

Primary isolation of the catecholamines

The instability of the catecholamines with their risk of oxidation makes it desirable to acetylate them to the considerably more stable acetyl derivatives as soon as possible Acetylation is an unselective process however and a very large number of acetylated products are therefore obtained when a tissue extract is acetylated with radioactive acetic anhydride without previous purification This renders difficult isolation of catecholamine derivatives in subsequent chromatographic analyses Further radioactive acetic anhydride is expensive We have therefore found it necessary to introduce a primary step of purification The losses which this causes are corrected for by the addition of ^{14}C-tracers of the catecholamines early in the procedure

We tried Dowex 50 (Bertler Carlsson and Rosengren 1958) and Amberlite CG 50 (Smith and Weil-Malherbe 1962) but these (as other ion-exchangers can also be expected to do) posed considerable problems. Salts were eluted from the columns and after concentration of the eluate greatly interfered with the acetylation process causing a poor recovery Absorption on aluminium oxide (Lund 1949 Weil-Malherbe and Bone 1957) on the other hand was found more suitable for primary isolation of catecholamines. A batch procedure in accordance with the technique described by Anton and Sayre (1962) was used The advantage of this technique over column chromatography was that the volume of the eluent could be kept lower and the recovery was rather greater Absorption of the catecholamines took place at pH 8.3

An extra addition of EDTA and bisulphite as has been recommended by most authors to reduce the destruction at the higher pH did not affect the recovery of adrenaline and noradrenaline in this step Since it is known that bisulphite can react with catecholamines such that alcoholic hydroxyl groups are replaced by sulphonic acid groups (Schroeter *et al.* 1958 Higuchi and Schroeter 1959 Kalstha 1970) which could have a disturbing effect bisulphite have been excluded from our method The extra addition of EDTA has been retained on account of its buffering effect which facilitates pH adjustments. EDTA can greatly interfere with the acetylation but no EDTA residues seem to pass over into the final eluate from the aluminium oxide

A study of the recovery of radioactively labelled catecholamines from tissue

extracts after absorption on alumina at different pH values was performed since Chang (1964) had reported a considerably lower optimal pH than used previously by most authors Under our experimental conditions absorption at pH 8.2 8.6 was found to give the best recovery however In a recently published paper Drell (1970) has shown that the molarity and ionic composition of the solution greatly influence the pH which gives optimal absorption on aluminium oxide columns This would seem to explain the variations in optimal pH previously reported by other authors.

During the pH adjustment magnetic stirring was avoided since this was found to cause considerable destruction of the aluminium oxide granules by grinding This resulted in very high amounts of aluminium residues in the eluate which interfered with the subsequent acetylation To overcome this problem round bottom flasks which were rotated manually but vigorously during the pH adjustment were used

Different methods to eliminate interfering substances by washing the aluminium oxide after absorption of the catecholamines were studied It was found especially in studies of brain tissue that the acetylation yield could be increased if the alumina was washed by shaking not only with water but also with some organic solvent that might remove lipids which had become absorbed by the aluminium oxide Absolute ethanol and acetone as suggested by Franklin and Mayer (1969) were used for this purpose No losses of catecholamines from the aluminium oxide take place on washing with these substances according to tracer experiments with ^{3}H-noradrenaline

Various eluents were also studied Acetic acid hydrochloric acid and perchloric acid have been used by most workers (Anton and Sayre 1962 Weil-Malherbe and Bone 1952) and in the THI fluorometric method the choice of eluent seems to affect the blank values We found hydrochloric acid and perchloric acid unsuitable since on subsequent freeze-drying of the eluate these caused very high acidity with partial destruction of the catecholamines (Roberts 1966 Forrest *et al.* 1970) In our studies with radioactively labelled catecholamines which were freeze-dried in dilute HCl, up to 30% of the catecholamines were destroyed in some cases. 0.5 N acetic acid on the other hand appeared ideal The elution from alumina is good and at the subsequent freeze-drying it gives an azeotropic mixture with water at a low concentration so that there is no risk of destruction of the catecholamines by concentration of the acid We found however that unlabelled acetyl groups disturbed the acetylation giving falsely low results Thus in spite of volatilization of the acid on freeze-drying prior to the acetylation acetyl groups appeared to remain probably bound to aluminium ions or some other non-volatile substance (see p 29). Therefore acetic acid which would seem ideal for use in freeze-drying of

catecholamines was found to be unsuitable in studies with acetylation Of the other acids which we studied formic acid was found the most satisfactory It is true that this acid concentrates on freeze-drying but according to our studies with labelled catecholamines which were freeze-dried in formic acid solutions and then cromatographed it caused no appreciable destruction of the catecholamines. In order for the formic acid not to dissolve too much alumina, the concentration must not be too high (see p 24). For effective elution from the alumina, however the pH of the eluate must be less than 4 A concentration of 0 1 N formic acid was found to give optimal results

If aluminium oxide is used as the primary separation step the applicability of the method is limited to substances of catechol structure In order to find a possibility of determining several catecholamine metabolites and also other monoamines without catechol structure more unspecific isolation steps were studied. The butanol technique for extraction of monoamines from tissues, described by *Shore* and *Olin* (1958) was found to be far too unselective in that several interfering substances greatly disturbed the recovery at the subsequent acetylation

Sephadex has been used relatively little as an isolation step for catecholamines (Marshall 1963 Cohen Bralet and Janiec 1968) Marshall used Sephadex G 25 (column 2 x 25 cm) for separation of catecholamines from plasma. We made preliminary studies using Sephadex 15 with columns of moderate length (0.8 x 10-?0 cm) which were eluted with 0 1 N acetic acid or 0 1 N formic acid When acetic acid was used the major proportion of the catecholamines was eluted in a satisfactorily small volume with sufficient retardation for residual large molecules to be separated from perchloric acid tissue homogenates. However in studies of brain tissue in particular acetylation of catecholamines in the freeze-dried eluate from Sephadex columns gave a rather poor yield The reason for this is not quite clear Marshall showed that a substance with absorption at 280 mμ was eluted together with the catecholamines after passage of plasma over a Sephadex bed It is conceivable that also residual perchlorate may cause interference even when the perchloric acid extract is partly neutralized with potassium carbonate before its passage through Sephadex

Liquid ion-pair extraction is another interesting technique for isolation of monoamines Temple and Gillespie (1966) described the solvent extraction of several amines from aqueous solutions with the aid of the ion-pairing compound di-(2-ethylhexyl) phosphoric acid (DEHP) The method has been used by Boon and Mace (1969) in determinations of adrenaline in formulated products. They showed that adrenaline could be essentially completely extracted from aqueous solutions with 1% DEHP in chloroform (vol/vol) and

that it could then be analysed directly by gas chromatography after silylation.

In a preliminary study with this technique using radioactively labelled catecholamines, we found that adrenaline and noradrenaline could be extracted into chloroform from perchloric acid tissue homogenates in the presence of DEHP (1%) at pH 7.8 By extraction of the organic phase with 0 1 N formic acid the amines could readily be transferred to the aqueous phase again After freeze-drying of the aqueous phase the amines were dissolved in 200 μl 1 mM HCl and acetylated by the triacetylation technique The reaction mixture was extracted with chloroform and chromatographed in system S_1 We found then that only 60-65% of the activity in the chloroform phase after acetylation migrated in the triacetate spot About 35% of the activity was found at the base of the chromatogram No attempts have yet been made to identify the unknown spot at the base of the chromatogram Whether 10 or 100 μmol acetic anhydride was used for acetylation did not affect this result It thus seems as if some of the catecholamines were altered in the isolation step so that they could no longer be converted to triacetates. However with an over all recovery of about 50% as triacetate the method is well worth further study and may be of great importance as a primary isolation step especially for monoamines that cannot be isolated over alumina.

Freeze-drying

In order to attain effective acetylation of the catecholamines with the use of as little acetic ^{3}H anhydride as possible the volume of the eluate from the alumina must be decreased In this process however most acids will be concentrated which as pointed out above may lead to destruction of the catecholamines. On freeze-drying of catecholamines in 0 1 N formic acid a study using radioactively labelled noradrenaline or adrenaline revealed no losses and no appreciable destruction At chromatography after the freeze-drying 95 98% of the activity was obtained in the catecholamine spot and the remainder was distributed evenly over the plate without any definitely identifiable individual peaks.

Acetylation

Acetylation of catecholamines has been studied extensively by Bretschneider (1946) He acetylated adrenaline with acetic anhydride in pyridine solution and obtained a tetraacetyl derivative i.e. acetylation of the amino and all three hydroxyl groups. Bretschneider's studies were conducted on a preparative scale but the procedure corresponds to that used successfully by Kliman and

Peterson (1960) among others for acetylation of steroids in a double-isotope technique Another method was described by Welsh (195?) here acetylation was performed with acetic anhydride in an aqueous solution in the presence of an excess of $NaHCO_3$ whereby triacetyl derivatives of adrenaline and noradrenaline were obtained the alcoholic hydroxyl group on the β-carbon atom remaining free We have conducted extensive studies of Bretschneider's tetraacetyl and Welsh's triacetyl technique in an attempt to find optimal conditions especially with respect to the amount of acetic anhydride required Both methods were found highly suitable for use on a microscale at which in both cases an essentially uniform product was obtained We found that the triacetyl technique required less acetic anhydride 10-?0 μmol per sample in acetylation of tissue extracts as compared with about 100 μmol for the tetraacetyl technique Further the former technique was rather more rapid and we therefore choose this for routine use The tetraacetyl technique has its value for corroboration of the results of the triacetyl technique however as will be discussed below Optimal conditions for the technique of tetraacetylation will be discussed in part 2

1 Triacetylation procedure

After concentration of the eluate by freeze-drying the catecholamines are dissolved in 200 μl of 1 mM HCl in order to give a stable solution For the acetylation we add the freshly distilled acetic ^3H anhydride solution (20% vol/vol in benzene) and immediately afterwards $NaHCO_3$ in small portions until gas development in the sample ceases A study of the amount of acetic anhydride needed when pure catecholamines were acetylated in 200 μl 1 mM HCl showed that a concentration of 0.01 M (0 1% vol/vol) of acetic anhydride in the reaction mixture was necessary for a satisfactory triacetate recovery (see fig. 3) However for the acetylation of extracts that first had been isolated on alumina a higher concentration of acetic anhydride was needed This will be further discussed below The concentration of acetic anhydride was found to be more important than the mole excess acetic anhydride over catecholamine The minimal concentration of acetic anhydride necessary for triacetylation was equal for adrenaline and noradrenaline

2. Effect of pH

For the recovery in the acetylation reaction we found the pH of the reaction mixture to be of great importance An experiment was performed in which 1 μg of noradrenaline was dissolved in buffer solutions of different pH and

acetylation took place with 20 µmol acetic anhydride The triacetate formed was extracted in chloroform and was determined after the first chromatography on silica gel in chloroform acetic acid 2 1 At a pH of 7 4 only 26% triacetate was obtained while pH 8.2 gave a maximal recovery of 90% In order to reduce the risk of destruction of the catecholamines before the acetylation when a buffer with a high pH was used borax buffer was studied It is well known that catechols can form a complex with the borate ion at an alkaline pH and in this way become protected from destruction (McOmie 1963) We found that within the pH range studied this complex formation did not prevent acetylation to triacetate Thus it would seem an advantage to use borax buffer at pH 8.2 in acetylation of catecholamines Acetylation at pH 7.8 which is attained in a saturated bicarbonate solution was found however to give such a good recovery – 80-85% – that we have continued to use this technique for routine

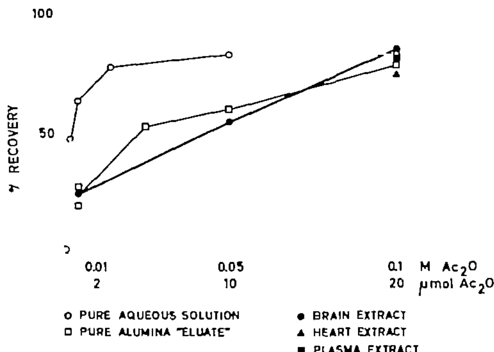


Fig. 3 Recovery from biological extracts, pure alumina eluates and pure aqueous solutions of ^{14}C-adrenaline (1 µg) as triacetate after triacetylation with varying amounts of acetic anhydride. The biological extracts and the pure alumina eluates were processed according to "Methods" except that the latter contained no tissue and that ^{14}C-adrenaline in all samples was added immediately before the acetylation. In the pure aqueous solution samples pure ^{14}C-adrenaline was acetylated in 1 mM HCl. The per cent recovered ^{14}C-activity after chloroform extraction and chromatography in S_1 is given.

purposes. No difference in the minimal amount of acetic anhydride required was noted between acetylation in borax buffer and in bicarbonate solution However recent experiments indicate that if a sample contains aluminium residues, more acetic anhydride is required for the acetylation if $NaHCO_3$ is used than if the acetylation is performed in a phosphate buffer (pH 8.0) (see p 25).

3 *Interfering factors*

More acetic anhydride had to be used for acetylation of catecholamines in tissue extracts than for acetylation in pure aqueous solutions. As has been described above different primary isolation steps have been studied The low recoveries obtained when catecholamines were acetylated in tissue extracts after the butanol extraction technique described by Shore and Olin (1958) and after Sephadex passage probably depended chiefly on residues from the tissue itself This may reduce the amount of available acetyl groups in the reaction mixture When instead ion-exchange resins or alumina were used as primary isolation steps, the interference with the acetylation was found to depend on the isolation step itself Ion-exchange resins posed considerable problems with disturbing salts when the eluates were concentrated Isolation on alumina gave the best result although even here more acetic anhydride had to be used than when acetylation was performed in pure aqueous solutions (see fig 3). Ion-pair extraction which we up to now only have studied briefly also seems promising but as some catecholamines were altered in this isolation step further studies must be performed

When alumina is used as the primary isolation step a gel is formed when the pH is raised by the addition of $NaHCO_3$ (which happens in connection with the acetylation). This gel forms even when alumina is shaken with pure 0 1 N formic acid and the supernatant is brought to pH 7.5 with $NaHCO_3$ The same happens with other acids. The gel therefore very probably is an aluminium hydroxide or a basic salt It is well known that aluminium hydroxide absorbs catecholamines from alkaline solutions (Shaw 1938) this might conceivably prevent complete triacetylation if small amounts of acetic anhydride are used A rough negative correlation has been found between the gel formation and the recoveries after triacetylation although it cannot be excluded that other substances not in gel form also may interfere

In order to obtain high recoveries after triacetylation the following factors were found to be of importance

a. The brand of alumina. Different brands of alumina gave differences in the acetylation yield. This correlated to some extent with the gel formation The

best results were obtained with Woelm Neutral Grade I The results presented in this paper were obtained with this alumina.

b An adequate pretreatment of the alumina can increase the triacetate recovery and reduce the gel formation Table I shows an experiment where alumina after different pretreatments was shaken with formic acid as in elution. The "eluates" were freeze-dried and dissolved in 200 µl 1 mM HCl. One µg ^{3}H-noradrenaline was added to each sample and triacetylation was performed with *0 025 M* acetic anhydride *The highest recovery as triacetate and least gel* formation was obtained after refluxing of the alumina in 2 N HCl followed by neutralization to pH 3-4 according to Anton and Sayre (1962) Washing of the alumina for several hours in formic acid did not reduce the gel formation compared to untreated alumina and the acetylation yield was only slightly higher

c. Too high acidity should not be used for elution of the alumina. In the experiment presented in table I the HCl-washed alumina was "eluted" with formic acid of three different strengths. In the sample where 0.5 N formic acid was used very much gel formed and an extremely low recovery was obtained On the contrary 0.05 N formic acid was found to give almost no visible formation of gel and recovery only slightly lower than the 80-90% obtained in pure aqueous solutions. However in order to make sure that the pH of the eluate is low enough to give sufficient elution of the catecholamines from alumina we have found it necessary to use 0 1 N formic acid as eluent This causes more gel to be formed and makes the minimal amount of acetic anhydride required somewhat higher

d. The contact time between the alumina and the eluate should not be too long. Elution of ^{14}C-adrenaline absorbed on alumina was performed in one

Table I

T acetylation f 1 µg ^{3}H-noradrenaline with 0.025 M acetic anhydride in 1 mM HCl solutions of freeze-dried "eluates" from alumina. Varying concentrations of formic acid were used for elution of the alumina, which was pretreated in different ways. The per cent ^{3}H-noradrenaline recovered as triacetate after extraction in chloroform and chromatography is given.

Pretreatment of alumina	Formic acid for elution	% triacetate recovery
Untreated	0.05 N	45
Untreated	0 1 N	6
0.1 N HCOOH washed	0.05 N	49
Conc HCOOH washed	0.05 N	57
HCl washed	0.05 N	77
HCl washed	0 1 N	50
HCl washed	0.5 N	3

group of samples by gentle shaking for ? times 1 min with 0 1 N formic acid and in another by vigorous shaking for 2 times 10 min The recoveries of ^{14}C adrenaline in the eluates were equal At the subsequent acetylation less gel formed in the former samples and the recoveries of ^{14}C adrenaline as triacetate were somewhat higher

e During the absorption of catecholamines on alumina magnetic stirring should be avoided as this causes considerable destruction of the granules extensive gel formation and low recoveries (see p 18)

Despite these precautions about 10 times more acetic anhydride is required for triacetylation of catecholamines in alumina eluates than in pure aqueous solutions In order to reduce the blanks caused by acetic ^{3}H anhydride in our method it should be of interest to further reduce the amount of acetic anhydride required A study of this problem is in progress. We have for instance tried to reduce the amount of alumina. 50 mg alumina could be used instead of 400 mg and still give a satisfactory recovery of catecholamines from perchloric acid extracts However this did not give any higher recoveries after acetylation.

The use of aluminium complexing agents has been more promising We have performed a preliminary study with cupferron acetylacetone and 8-hydroxy quinoline These substances form aluminium complexes which can be extracted with chloroform from the formic acid eluate before freeze-drying and acetylation In preliminary experiments we have obtained somewhat varying results but it seems that both cupferron and acetylacetone can reduce the amount of acetic anhydride required No gel formation could be observed in these samples when the pH was raised Further studies must be performed however to find out the optimal amounts of complexones and the optimal pH for the extraction of the aluminium complexes

Another promising possibility to reduce the interference caused by aluminium residues is to perform the acetylation in a phosphate buffer pH 8.0 Preliminary experiments indicate that this increases the triacetate recovery when small amounts of acetic anhydride are used

4 *Acetylation in biological materials*

In order to see whether the tissue itself interferes with the acetylation reaction extracts in perchloric acid of 0.5 g rat brain were passed over alumina, eluted in 0 1 N formic acid and freeze-dried ^{14}C-adrenaline was added and acetylation was performed in 200 µl 1 mM HCl with varying concentrations of acetic anhydride These samples were compared with a group of samples treated in the same way but with no tissue present The recoveries of ^{14}C-adrenaline as

triacetates (after extraction in chloroform and chromatographic isolation on a silica gel plate) are shown in fig. 3 (p 22) It is obvious from comparison of the tissue + alumina line and the alumina-only line that the tissue itself interfered only little with the acetylation. Recoveries increased with the amount of acetic anhydride However the need for much acetic anhydride is not due to a consumption of the acetic anhydride the lines do not intercept the positive x-axis. It is therefore more probable that catecholamines bind to aluminium hydroxide gel or some basic aluminium salt in the reaction mixture

The figure also shows the recoveries obtained when 0.5 g rat heart and 10 ml human plasma were treated like the brain samples and acetylated with 0 1 M acetic anhydride The recoveries were about equal to that obtained with the brain tissue

Evidently a minimal amount of 15 20 μmol (about 1.5 2 μl) acetic anhydride which gives a concentration of about 1% (vol/vol) in the reaction mixture is necessary for recoveries around 70 80% if aluminium salts are not removed

5 pH adjustment after acetylation

The acetylation of the catecholamines in aqueous solution with acetic anhydride in the presence of $NaHCO_3$ in excess takes place in less than one minute In order to ensure that the excess of acetic anhydride has been inactivated we usually allow the samples to stand for about 1/2 hour before addition of carrier triacetate and extraction But if during this time the reaction mixture is left at pH 7.8 the triacetate formed will be slowly hydrolyzed (After 1 hour the triacetate is about 90% of the original level and the following day the recovery is less than 10%) Therefore the samples are acidified some 5 min after the acetylation with N HCl to a pH of about 3 4 tested with pH paper The pH must not be too low however as the catecholamine triacetate can also be destroyed at a highly acid pH (see p 28)

6. Acetylation on alumina

We have attempted to simplify the acetylation technique by allowing the primary absorption of catecholamines to take place on a minimal amount of alumina and then carry out the acetylation directly with no preceeding elution of the catecholamines However the recovery was very poor Franklin and Mayer (1968) reported that triacetylation can be done with the catecholamines still absorbed on alumina. For this, however they used acetic anhydride in pyridine solution according to Bretschneider (1946) In our studies with this

technique the recovery was also poor and mainly tetraacetylated catecholamines were obtained The technique required considerably more radioactive acetic anhydride with all the disadvantages which this involves.

Extraction

The extraction of the catecholamine triacetate formed takes place with chloroform Adrenaline dopamine and isoprenaline triacetate have relatively low solubility in water and can easily be extracted by an organic solvent Noradrenaline triacetate is somewhat more polar and more soluble in water however and therefore repeated extraction with a high excess of chloroform is necessary The water phase at this stage is about 0.5 ml and 3 x ? ml chloroform are sufficient for extraction even of noradrenaline triacetate Dichloromethane has been preferred by several authors who have used the triacetylation technique (Laverty and Sharman 1965 Crawford and Yates 1970) but we found no advantage over chloroform in our studies.

As mentioned previously it is necessary partly for reasons of cost to use as little radioactive acetic anhydride as possible in the acetylation procedures With the amounts which we use the acetylation is not as a rule complete and some di- and monoacetylated products are also obtained With the double isotope technique however a total recovery is not necessary and as seen in table II the triacetate formed is well separated from the other products Chromatography of the chloroform phase after concentration under an air stream shows that 95-98% of this activity can be found in the triacetate spot Chromatography of the residual aqueous phase shows that the remaining

Table II

Distribution of the ^{14}C-activity in per cent of total recovery in silica gel chromatograms following triacetylation of 0.5 μg ^{14}C-adrenaline (sample A) and ^{14}C noradrenaline (sample N) with 0 075 M unlabelled acetic anhydrid by the standard procedure. The triacetates were extracted in 3 x 2 ml chloroform The pooled chloroform and the water phases were then chromatographed separately in chloroform acetic acid (sample A 2 1 sample N 1 1). Total recovery means total activity recovered in chloroform plu water phase

Note absence of disturbing peak in chloroform phase

Sample	Phase	Fraction in cm from starting line							
		0-2	2-4	4-6	6-7	7-9	9-10	10-12	12 14
A	chloroform	0 54	0.22	0.33	0.54	70.22	0 92	0.27	0.27
	water	14.85	3 94	2.99	2 17	1 74	0.01	0.02	0 01
N	chloroform	0 70	0 48	0.50	0 99	65.04	0.53	0 43	0.00
	water	8.42	4 43	8 15	5 32	4 78	0.07	0.07	0.09

activity here migrates with quite different Rf values Tests with catecholamines of lower degrees of acetylation indicate that these slow moving fractions mainly consist of incompletely acetylated material.

Besides the slow moving partially acetylated amines there also is a small spot migrating in front of the triacetates. It has been verified by mass spectrometry *that this small amount of radioactivity consists of tetraacetate* Usually however it comprises less than 1% of the triacetate formed

Stability of triacetylated catecholamine derivatives

For the triacetyl derivatives formed to be isolated by repeated chromatographies they must be sufficiently stable Problems with destruction of catecholamine triacetates have been reported among others, by Waldi (1962) who stated that adrenaline triacetate solutions were only stable for a few hours We found the stability to be fairly good in methanol and chloroform At +4 C a few per cent per week were destroyed In aqueous solutions the catecholamine triacetates were found to be essentially stable in the pH range 2 7 for at least some hours at room temperature Outside this range they are quickly hydrolyzed

The stability at chromatography in the different systems which we use was studied with purified catecholamine triacetates which were chromatographed with the two-dimensional technique In thin-layer chromatography on silica gel in system S_1 S_3 (see table VI) 99 100% of the activity on the plate was found in the triacetate spot (see table III). On chroma graphy in system S_2 and S_7 on silica gel and also the paper chromatography systems used (P_4 and P_6) the recovery was 95 99% The Rf values and the $^3H/^{14}C$ ratios in double isotope studies showed that the radioactivity outside the triacetate spot is probably mainly diacetate (see table III) Thus some destruction of the

Table III

Distribution of ^{14}C-activity in silica gel chromatograms following two-dimensional chromatography of ^{14}C-adrenaline ^3H-triacetate in system S_7 (1) and S_3 (2) The activity was analysed fractionally from starting line to front in the second chromatography direction According to the $^3H/^{14}C$ ratio the activities in fraction 6 7 7 8 and 8 9 were unaltered triacetates The variation in the ratio from 5.88 to 5.42 is du to isotope fractionation (see below) The activity in fraction 4 6 depends on diacetate according to the ratio

	Fractions in cm from starting line							
	0-2	2-4	4-6	6-7	7-8	8-9	9 11	11 13
% ^{14}C-activity	0 05	0 07	0.81	0 65	96 21	2.20	0.01	0.00
$^3H/^{14}C$ ratio			3.80	5.88	5 76	5 42		

triacetylated catecholamines occurs in certain chromatographic systems, but this does not affect the result since the diacetate formed migrates in the chromatogram with good separation from the main triacetate spot

On some occasions during chromatographic purification a considerably higher destruction of the triacetate occurred erratically in isolated samples while duplicate samples under identical conditions were completely unaffected The reason for this destruction has not been found with certainty We made a study to find out whether remnants of dish-washing detergents (Liqui-nox® RBS-25®) or potassium hydroxide which were used for cleaning of glassware could contribute to the destruction if the glassware was incompletely rinsed but no such effect was found

In paper chromatography a study was also made of the effect of prolonged washing of the chromatography paper but this had no influence on the sometimes surprisingly high destruction of the catecholamine triacetates

Some destruction of the triacetates can be accepted since the method is not dependent upon the absolute recovery but the erratic large losses sometimes encountered pose a problem which has not yet been solved

Disturbing effects of unlabelled acetic acid

1 During acetylation

In the beginning of our studies the acetylation of catecholamines to triacetates was carried out with 0.5 N acetic acid as solvent We then found that the $^{3}H/^{14}C$ ratios in ^{3}H-triacetylated ^{14}C-catecholamines were considerably lower than might have been expected from the specific activity of the radioactive substances. One possible explanation for this is that unlabelled acetyl groups from the solvent might have become incorporated in the catecholamine triacetate molecule during the acetylation instead of ^{3}H-labelled acetyl groups from the acetic anhydride When catecholamines that have been absorbed on alumina are acetylated it is possible that residues of aluminium may catalytically cause this effect However it was also observed on acetylation of catecholamines that had not passed alumina.

When the triacetylation was performed in weak HCl or formic acid solution a higher $^{3}H/^{14}C$ ratio corresponding more closely to that calculated theoretically was obtained (see table IV sample 1 and 2)

In further studies of factors that might disturb radioacetylation it was found that a lower $^{3}H/^{14}C$ ratio than calculated theoretically also could be obtained on triacetylation in HCl if the catecholamines had been previously eluted from aluminium oxide with acetic acid freeze-dried and dissolved in HCl before the

acetylation (see table IV sample 3 and 4) Evidently acetyl groups could remain bound in the sample despite freeze-drying of the acetic acid eluate

2. During storage and chromatography

The possibility that exchange of ^{3}H-labelled acetyl groups from catecholamine triacetate can take place with acetyl groups from solvents on storage or chromatography in acetic acid was also studied It was found that incorporation of unlabelled acetyl groups into the catecholamine triacetate molecule could apparently occur only under extreme conditions For example a small proportion of noradrenaline triacetate was converted to tetraacetate on storage in concentrated acetic acid at 70°C for 24 hours, but no exchange of already bound acetyl groups in the molecule with acetyl groups from the solvent was observed Similarly repeated chromatographies of double-labelled acetylated catecholamines in acetic acid chloroform (1 1) led to a constant ^{3}H/^{14}C ratio which would not have been the case if exchange of acetyl groups had taken place during the chromatographies (see table V)

Table IV

Evidence for disturbance of radioacetylation by unlabelled acetic acid The ^{3}H/^{14}C ratios in successive chromatographies, in different systems, after triacetylation of ^{14}C-catecholamines with acetic ^{3}H anhydride are given.

Sample 1 ^{14}C-adrenaline acetylated in 200 µl 0.5 N acetic acid.

Sample 2 ^{14}C-adrenaline acetylated in 200 µl 1 mM HCl

Sample 3 ^{14}C-noradrenaline eluted from alumina in 0.5 N acetic acid, freeze-dried and acetylated in 200 µl HCl.

Sample 4 ^{14}C-noradrenaline eluted from alumina in 0 1 N f rmic acid freeze-dried and acetylated in 200 µl HCl.

Sample	^{3}H/^{14}C ratio after chromatography no 1	2	3	4	5	6	Expected ratio
1	5 78	5.05	5.29	4 99	4.86	4.98	6 10
2	6.82	6.05	6 12	6.06	6 10	6.07	6 10
3	4.61	3 68	4 06	3 79	3 83		5 75
4	6 40	5 42	5 91	5.56	5.65		5 75

Table V

The ^{3}H/^{14}C ratio in successive chromatographies on silica gel in acetic acid chloroform (1 1) after acetylation of ^{14}C-adrenaline with acetic ^{3}H anhydride. Note that constant ratios are obtained

	Chromatography no 1	2	3	4	5	6	7
^{3}H/^{14}C ratio	5 77	5 35	5.21	5 21	5.05	5.05	5.05

Purification and separation of catecholamine triacetates

Since acetylation is a very unselective reaction the acetates formed must be carefully separated from other ^3H-labelled products In order to remove acetic ^3H anhydride the acetates produced are extracted into chloroform. On simple extraction with chloroform however a fair amount of acetic ^3H acid is also taken up in the chloroform phase Extensive washing of the chloroform phase with water should reduce this ^3H background before the chromatographic separation but noradrenaline triacetate in particular is fairly soluble in water and considerable losses can therefore occur in this process Conversion after the acetylation of the excess of acetic ^3H acid with ethanol to more highly volatile ethyl acetate which was then removed by freeze-drying, gave no better results than simple chloroform extraction of the reaction mixture after the acetylation It turned out that repeated chromatography in combination with the use of as little acetic ^3H anhydride as possible was the best way to get rid of the impurities

1 Different chromatography systems

In order further to separate and purify the different catecholamine triacetates several different chromatographic systems are required A number of these are summarized in table VI A modified Bush C system (table VI P_4) was introduced by Goldstein Friedhoff and Simmonds (1959) for separation of different catecholamine triacetates In a recent study of tetraacetates Matthias and Liemann (1970) used a Zaffaroni system (toluene:ethyl methyl ketone 3 1 on formamide impregnated paper) which we have further modified (P_6) Two other paper systems for catecholamine triacetate have been described by Hagopian Dorfman and Gut (1961) and by Lavarty and Sharman (1965) Finally Stern Franklin and Mayer (1967) have described some systems for the separation of adrenaline and noradrenaline triacetate on glass fibre paper which have not been tried by us.

When the present study was started relatively few chromatographic systems using silica gel had been reported A combination of paper partition chromatography and the more rapid absorption chromatography on silica gel was considered desirable however and considerable work was therefore put into the development of suitable silica gel systems. Two such systems for triacetates have been described by Waldi (1962) (S_2 and S_8) We developed a number of systems of which the best are shown in table VI Forest and Heacock (1969) have recently described several systems for separation of acetylated adrenaline derivatives of which one S_7 is identical with one of ours

Table VI
Rf values for catecholamine tri- and tetraacetates Na noradrenaline A adrenaline DA dopamine and Iso isoprenaline

System no	Stationary phase	Chromatography system	Rf-values triacetate NA	triacetate A	triacetate DA	triacetate Iso	tetraacetate NA	tetraacetate A	tetraacetate Iso	Remarks
S_1	Silica gel	Chloroform acetic acid (2 1)	0.38	0.52	0.61	0 72	0.50	0 74	0.87	
S_2	Silica gel	Chloroform methanol (9 1)	0.36	0 46	0.52	0.56	0.51	0.62	0.66	
P_4	Paper	Toluene ethyl acetate methanol water (10 1.5 5)	0.05	0.31	0 47	0.83	0 43	0 72	0 77	Organic phase Front migrates 10cm/h
S_5	Silica gel	Chloroform cyclohexane acetic acid methanol (5.3 1 1)	0.31	0 42	0.54	0.58	0 45	0.55	0 65	
P_6	Paper	Toluene ethyl acetate (3 1) (formamide-saturated)	0.02	0.06	0 10	0.34	0.07	0.28	0 47	Formamide impreg. paper Front migrates 13 cm/h
S_7	Silica gel	Chloroform pyridine (9 1)	0.30	0.56	0 44	0 76	0 46	0.80	0.90	
S_8	Silica gel	Chloroform acetic acid (4 1)	0 23	0.37	0 42	0.52	0.32	0.54	0 70	
S_9	Silica gel	Chloroform formic acid (9 1)	0 17	0.25	0.30	0.28	0.23	0.35	0.33	
S_{10}	Silica gel	Acetone:pyridine (4 1)	0.67	0.69	0 78	0.81	0.80	0.83	0.87	
S_{11}	Silica gel	Acetone chloroform (4 1)	0 44	0 44	0 44	0.53	0.53	0.52	0.56	
S_{12}	Silica gel	Diisobutylketone:pyridine (5 1)	0 11	0 16	0 14	0.27	0 15	0.22	0.30	
S_{13}	Silica gel	Chloroform acetic acid :diisobutylketone (9 1 1)	0 15	0.29	0.22	0.37	0.21	0 46	0.56	

2 Choice of system

As the first chromatographic step in the triacetylation method we have found chloroform:acetic acid (2 1) (S_1) to be particularly suitable In this system residual acetic ^3H acid migrates to the front and is effectively separated Noradrenaline adrenaline dopamine and isoprenaline triacetates also separate well They are then purified individually in a succession of systems as described on p 11

System P_4 gives good separation without equilibrating the paper with the aqueous phase prior to the chromatography If this system is used on TLC cellulose the plates have to stand over night in the aqueous phase atmosphere before chromatography in the organic phase to prevent extensive tailing. Therefore TLC cellulose was less suitable Paper chromatography in the Zaffaroni system (toluene:ethyl methyl ketone 3 1) showed tailing which however could be reduced if ethyl acetate was used in stead of ethyl methyl ketone (P_4)

3 Localization of the spots

Localization of the spots in the chromatograms must be done without destruction as occurs, for example with strongly basic stains. Since the $^3H/^{14}C$ ratio is determined by liquid scintillation counting the eluate should also produce minimal quenching

Identification of the substances on silica gel is easily performed by the use of silica gels containing a fluorescent substance (HF_{254} acc to Stahl) In paper chromatography different staining methods have been studied and we have found sodium fluorescein the most suitable The fluorescence is quenched in the spot. Only very small amounts of fluorescein are eluted together with the catecholamine triacetates if chloroform is used as the eluent They do not interfere with the radioactive determination of the $^3H/^{14}C$ ratio and disappear in subsequent silica gel chromatography However we prefer methanol as an eluent for the triacetates when paper chromatography in system P_4 is used since it gives better recovery It has the disadvantage however that it dissolves considerable amounts of fluorescein Therefore we usually prefer to spray just a narrow strip of the chromatogram This strip is then used for identification of the spot, and the rest eluted with methanol The test strip is cut out from the centre of the chromatogram and is sprayed with sodium fluorescein followed by ethylenediamine When these two stains are used together the spot appears red in visible light and as a considerably increased quenching in UV light (254 mμ) compared to that obtained with sodium fluorescein alone However the triacetates are destroyed in this part of the chromatogram

It is very important to map the whole extent of the spot as exactly as possible in order to avoid errors due to isotope fractionation (see below) If the spot is regular and symmetrical it can be reliably identified from a small strip but if it is not part of the spot can be missed when it is cut out. Therefore after the spot has been cut out we always in critical experiments check, by spraying the rest of the paper that no part of the spot has been left Localization may of course also be performed with a chromatogram scanner but this has not been available in our experiments

4 Elution of the spots

In the elution of silica gel chromatograms methanol and chloroform give similar yields, approximately 90% of the radioactivity being found in the eluate For elution of paper chromatograms in system P_4 methanol is most effective If on the other hand formamide impregnated paper (P_6) is used chloroform is the eluent of choice since methanol also elutes formamide This complicates further chromatographic analyses because of its low volatility Residues of formamide also give high background values because of chemoluminiscence

5 Rf values for related acetylated compounds

Table VII gives Rf values for some catechol metabolites and other monoamines that were acetylated by the technique which we used for triacetylation of the catecholamines. Apart from those which we studied it is of course possible that

Table VII

Rf values after acetylation acc. to Welsh. DOPA 3 4-dihydroxyphenylalanine DOMA 3 4-dihydroxymandelic acid DOPAC 3 4-dihydroxyphenylacetic acid, VMA = 3-metoxy-4-hydroxymandelic acid HVA 3-methoxy-4-hydroxyphenylacetic acid MOPEG 3-metoxy-4-hydroxyphenylethylene glycol, M metanephrine NM normetanephrine 5HT 5-hydroxytryptamine The chromatography systems are described in table VI

System	*Rf-values after acetylation acc. to Welsh*								
no.	DOPA	DOMA	DOPAC	VMA	HVA	MOPEG	NM	M	5 HT
S_1	0 8	0 70	0 75	0.68	0 79	0.34	0.24	0.38	0.34
S_2	0.09	0 21	0.52	0.39	0.50	0.59	0.51	0.61	0.39
P_4	0 07	0.37	0.53	0.67	0.65	0 14	0 04	0 46	0 14
S_5	0 33	0.53	0.69	0 63	0 74	0.37	0 40	0 45	0.54
P_6	0 00	0 02	0 13	0.03	0.08	0.51	0.03	0 10	0.03
S_7	0 07	0 19	0 67	0.26	0.67	0.38	0.39	0.52	0.25

a large number of other substances were acetylated and had Rf values similar to those of acetylated catecholamines At low concentrations of catecholamines in tissues or when due to the effect of blocking agents metabolites have increased greatly the separation of catecholamine acetates from the metabolites can be difficult if only a few different chromatographic systems are used This is evident from a comparison of the Rf values in table VI and VII Other acetylated monoamines also such as serotonin may give rise to problems in separation from adrenaline and noradrenaline triacetate This shows the importance of a primary purification step before the fairly unselective acetylation reaction Absorption on alumina will remove metabolites and other substances without a catechol structure and also to a large extent catechol acids since these although absorbed on the alumina require stronger acid than the catecholamines for elution (Weil and Malherbe 1968)

The possibility of acetylating several monoamines and catechols indicates that a double isotope technique with an acetylation procedure might be employable also for quantitative determination of substances other than catecholamines. In order to reduce the complexity of the chromatographic separation procedure and the amount of acetic anhydride required a primary isolation step should be developed for the different groups of substances before acetylation It should be possible for example to further develop serotonin determination with acetylation in a double isotope method but this lies outside the primary aim of the study

Mathematics of the method

The amount of one catecholamine (x) in μg in a sample is calculated from the formula

$$x = a \cdot \frac{t_s}{t_I} \cdot \frac{r_s - r_{II}}{r_I - r_{II}} \tag{1}$$

Symbols.

- a = μg unlabelled catecholamine added to standard I
- t_s = cpm ^{14}C tracer added to sample
- t_I = cpm ^{14}C tracer added to standard I
- r = $^{3}H/^{14}C$ ratio of sample
- r_I = $^{3}H/^{14}C$ ratio of standard I.
- r_{II} = $^{3}H/^{14}C$ ratio of standard II
- t_{II} = cpm ^{14}C tracer added to standard II
- A_3 = specific activity of acetic ^{3}H anhydride (Ci/mol).
- A_{14} = specific activity of ^{14}C-catecholamine (Ci/mol)

M = molecular weight of catecholamine
e_3 = ^3H efficiency in channel 1
e_{14} = ^{14}C efficiency in channel 2

Formula (1) is derived from the following reasoning

Cpm ^3H in the initial sample that would have been bound by the unknown + tracer immediately after the addition of tracer

$$t_s \quad r_s$$

or in µmol acetic ^3H anhydride

$$\frac{t_s \quad r_s}{A_3 \quad e_3 \quad 2.22 \quad 10^6}$$

Since 1 µmol catecholamine combines with 1.5 µmol acetic anhydride the unknown + tracer in the initial sample are (µmol)

$$\frac{t_s \quad r}{1.5 \quad A_3 \quad e_3 \quad 2.22 \quad 10^6}$$

or in µg

$$\frac{M \quad t_s \quad r}{1.5 \quad A_3 \quad e_3 \quad 2.22 \quad 10^6}$$

Since the amount of tracer added to the sample in µg is

$$\frac{M \quad t_s}{A_{14} \quad e_{14} \quad 2.22 \quad 10^6}$$

the unknown x in µg is

$$x = \frac{M \quad t \quad r_s}{1.5 \quad A_3 \quad e_3 \quad 2.22 \quad 10^6} - \frac{M \quad t}{A_{14} \quad e_{14} \quad 2.22 \quad 10^6} \qquad (2)$$

The same reasoning for standard I and II gives

$$a = \frac{M \quad t_I \quad r_I}{1.5 \quad A_3 \quad e_3 \quad 2.22 \quad 10^6} - \frac{M \quad t_I}{A_{14} \quad e_{14} \quad 2.22 \quad 10^6} \qquad (3)$$

$$0 = \frac{M \quad t_{II} \quad r_{II}}{1.5 \quad A_3 \quad e_3 \quad 2.22 \quad 10^6} - \frac{M \quad t_{II}}{A_{14} \quad e_{14} \quad 2.22 \quad 10^6} \qquad (4)$$

If the values for A_3 and A_{14} are solved from equation (3) and (4) and inserted into equation (2) formula (1) results.

From formula (1) one sees

a. The formula does not contain the following magnitudes specific activities of tracer catecholamine (A_{14}) and acetic anhydride (A_3) and the cpm tracer added to standard II (t_{II}) These therefore do not have to be known with high precision However the specific activities should be approximately known for reasons which will be discussed

b The amount of unlabelled catecholamine (a) used in standard I directly influences the result When solutions of different commercial catecholamine salts were assayed with our method small variations of the values were obtained indicating that the catecholamines were partly oxidized To avoid this the catecholamine used as standard should be purified repeatedly by crystallization before use in experiments where absolute values of x are important

c Absolute purity of the ^{14}C-catecholamine used as tracer is not essential if the tracers added to the standards (t_I and t_{II}) and added to the sample (t_s) are taken at the same time from the same stock solution

d. The proportion between the volumes of tracer solution used for t_I and t_s have to be accurately known since $\frac{t_s}{t_I}$ directly influences the result

e The amounts of tracer t and of unlabelled catecholamine (a) added to standard I influence the precision This is seen from the following

$$\frac{r_s - r_{II}}{r_I - r_{II}}$$

(part of equation 1) may be written as follows, where all magnitudes are in cpm

$$\frac{\dfrac{^3\text{H from x} + {}^3\text{H from } t_s}{^{14}\text{C from } t_s} - \dfrac{^3\text{H from } t_{II}}{^{14}\text{C from } t_{II}}}{\dfrac{^3\text{H from } a + {}^3\text{H from } t_I}{^{14}\text{C from } t_I} - \dfrac{^3\text{H from } t_{II}}{^{14}\text{C from } t_{II}}}$$

Evidently the effect of a random error in r_{II} is minimized if r_s is close to r_I The other way of minimizing the effect of random errors in r_{II} is to make r_s and r_I much larger than r_{II} Obviously r_{II} is determined only by the specific activities of the ^{14}C-tracer and the acetic ^{3}H anhydride In order to make r and r_I much larger than r_{II} one must therefore aim at high values for r and r_I This can easily be done with regard to r_I if a is made large enough But in the case of r_s the unknown amount (x) cannot be chosen at will Therefore the chemical

amount of tracer added to the sample (t_s $10^{-4}/e_{14}$ A_{14} 2.22) must be small enough. The lower limit is determined by the necessity of having enough ^{14}C-counts after all purification steps Evidently therefore the specific activity A_{14} and the efficiency of ^{14}C-counting (e_{14}) should be as high as possible if small unknown amounts of catecholamines are to be determined

If x is very small (r_s r_{II}) may still not be large enough to make the random errors of r_{II} negligible It is in this situation that it may be useful to make r_I similar to r_s Since x is unknown and r_s therefore cannot be predicted it can be worth the trouble (and the acetic ^3H anhydride) to run several standard I with different additions of a In the calculation one then uses the r_I-value that is closest to r_s

f The specific activity of acetic ^3H anhydride should be high enough to minimize the random error caused by ^{14}C in the ^3H-channel at liquid scintillation counting With the discriminator settings used by us, 14% of ^{14}C-counts appear in the ^3H-channel (the ^3H-count in the ^{14}C-channel was negligible). The ^3H-counts in the ^3H-channel should be around 10 times higher than the contribution from ^{14}C Our ^3H-efficiency (e_3) is 0.26 and ^{14}C-efficiency (e_{14}) 0.58 This gives

$$1.5 \quad 0.26 \quad A_3 \geqslant 10 \quad 0.14 \quad 0.58 \quad A_{14} \quad \frac{A_3}{A_{14}} > \sim 2$$

Thus the proportion between A_3 and A_{14} that should be used is around 2 It should not be much less and it does not pay to make it much larger The best specific activity of the ^{14}C-catecholamines that are available at the present time is about 60 Ci/mol Therefore acetic anhydride with about 100 Ci/mol is sufficient

Theoretical sensitivity

The limiting factors for the theoretical sensitivity are

1 There must be sufficient cpm in the final chromatogram to give reliable ^3H and ^{14}C-counts

2 The chemical amount of tracer added to the sample containing the unknown should not be much larger than the unknown itself (x) preferably it should be smaller

From our experience sometimes only 5% of the original ^{14}C-counts added to the initial sample are recovered in the final chromatogram spot Activities should preferably be at least 5 times background With a background in the ^{14}C-channel of at most 30 cpm and in the ^3H-channel of at most 60 cpm at least 5 30 20 = 3000 cpm ^{14}C and 5 60 20 = 6000 cpm ^3H should be present

as triacetate after the acetylation If ^{14}C-catecholamines (60 Ci/mol) are used this corresponds to an addition of 7 ng tracer (t_1) to the sample On the other hand 12 ng catecholamine (unknown x + chemical amount of tracer) is sufficient to give reliable ^{3}H-counts if acetic ^{3}H anhydride with 100 Ci/mol is used Thus, here the limit of the unknown x is not much less than 6 ng and requires the addition of about 6 ng ^{14}C-tracer Evidently therefore the specific activity of the ^{14}C-tracer is the limiting factor for the sensitivity and the magnitude of the unknown (x) should not be much less than the 7 ng tracer added to the sample

Exactly the same kind of reasoning applied to the case when ^{3}H-catecholamines (5000 Ci/mol) and acetic ^{14}C anhydride (100 Ci/mol) are used instead gives a smallest addition of tracer of 0 4 ng to get reliable ^{3}H-counting. However here the specific activity of the acetic ^{14}C anhydride is limiting and the total amount of catecholamine (unknown + tracer) in the initial sample must be at least 3 ng to give reliable ^{14}C-counts Thus the limit of the unknown x is not much less than 1.5 ng and requires the addition of about 1.5 ng ^{3}H-tracer However the cost per determination with acetic ^{14}C anhydride of such high specific activity is considerable (At present it is about 100 times more expensive than acetic ^{3}H anhydride of the same specific activity).

The use of ^{14}C-catecholamines labelled in more than one position evidently would increase the theoretical sensitivity This should also permit further purification of critical samples and thereby reduce the problem posed by high tissue blanks (see p 69).

The reader will have noticed that the above reasoning is based on very conservative estimates. As a matter of fact the final recovery sometimes is far better than 5% and the actual sensitivity accordingly increased It therefore is rational to repeat critical experiments several times, it happens that enough counts remain in one but not the other

Bromoacetic anhydride acetylation

Since the critical sensitivity of the method is limited by the relatively low specific activities of ^{14}C-catecholamines or acetic ^{14}C anhydride available at present in comparison with ^{3}H-labelled tracers or acetic ^{3}H anhydride some attempts were made to replace the ^{14}C isotope by another isotope with higher specific activity One possibility was the use of bromoacetic anhydride By neutron activation the bromine can be converted to ^{82}Br which should allow determination of very small amounts of bromine Using this technique it was possible to first determine the amount of ^{3}H in an aliquot of the sample and

then to perform a neutron activation analysis of the bromine However it was found difficult to obtain a homogenous product and a satisfactory recovery on acetylation of catecholamines with bromoacetic anhydride Different acetylation techniques were studied Acetylation according to the method of Welsh did not give the desired recovery and neither did tetraacetylation by the Bretschneider technique with pyridine as catalyst since precipitation occurred Acetylation in a dry environment with bromoacetic anhydride and with zinc chloride as catalyst gave a somewhat better recovery but no homogenous product was obtained Another problem was the background of bromine picked up during the chromatographies This was especially bad with silica gel. Since theoretically the technique should allow determinations of very small quantities of catecholamines it would seem of interest to attempt to develop it further even if in our hands it has not yet been of practical use

Chapter III
CRYSTALLIZATION AND SECOND DERIVATION

A constant $^3H/^{14}C$ ratio in successive chromatographies in different systems strongly indicates that pure catecholamine triacetate has actually been separated. In order to have further assurance of the absence of any ^3H-labelled impurity migrating with the same Rf value as the triacetate in question and thereby leading to a false result in the quantitative determinations, it sometimes is desirable to check the purity by further steps. This chapter deals with two such extra steps. One is crystallization: the $^3H/^{14}C$ ratio should remain constant in the case of purity. Another is second derivation to a new derivative with other chromatographic properties (for references, see review by Whitehead and Dean 1968).

Crystallization

Crystalline catecholamine triacetates have been described by Welsh (1955). In our own experiments we found that the most suitable technique was crystallization from acetone/ether or benzene/ether after seeding at –20°C. The catecholamine triacetates are characteristically microcrystalline and unfortunately the crystallization takes place very slowly. In attempts to shorten the time of crystallization by means of a large excess of ether or rapid cooling they precipitate very easily as oil. We found dl- and l-noradrenaline triacetate extremely troublesome. Even with adrenaline the technique is not ideal: the recovery is rather poor and the crystallization takes too long time for routine use. Table VIII shows an experiment where ^{14}C-adrenaline was triacetylated with acetic-^3H anhydride and purified by repeated chromatography until constant $^3H/^{14}C$ ratio. Two crystallizations (no. 1 from acetone, no. 2 from benzene) were then performed and the table shows that a true constant ratio had been obtained. Each crystallization needed 3 days at –20 C after addition of unlabelled adrenaline triacetate carrier. Thus, although crystallization of catecholamine triacetates is rather time-consuming and difficult, it can be used as an extra check of purity in certain more critical samples.

Second derivation

The technique of second derivation gives another possibility of ensuring that the individual catecholamine triacetates actually have been separated and may also lead to more rapid achievement of a pure product in chromatographic

Table VIII
$^3H/^{14}C$ ratios after chromatography of ^{14}C-adrenaline ^3H-triacetate in four different systems and after crystallization (1 acetone ether 2 benzene ether) Recoveries in per cent of the remainder after each preceding step

	Chromatography no				Crystallization no.	
	1	2	3	4	1	2
$^3H/^{14}C$ ratio	13.2	5.60	5.51	5 75	5.83	5.60
% recovery	62	80	81	79	55	30

separation procedures. The aim is to obtain a new derivative with other chromatographic properties but where the radioactively labelled groups in the original molecule have been retained in an unaltered state or reduced to a degree known in advance Second derivation has been used in other connections by several investigators working with double isotope techniques e.g by Kliman and Peterson (1960) for steroid determination Theoretically catecholamine triacetates with a free OH group on the β-carbon atom should be well suited for continued derivation, using the remaining OH group

1 Second acetylation

We found it possible by further treatment of adrenaline and noradrenaline triacetate with unlabelled acetic anhydride according to Bretschneider to achieve an almost complete conversion of triacetate to tetraacetate (see table IX).

Table X presents some experiments in which second acetylation was performed in an attempt to prove that noradrenaline and adrenaline triacetate had indeed been separated from other ^3H-labelled products Since in two of the samples a small decrease in the ratios was observed after tetraacetylation one might suspect that a small exchange of acetyl groups had taken place Therefore a study was performed where ^{14}C-catecholamine ^3H-triacetates (chromatographed until constant ratios) were repeatedly treated with unlabelled acetic anhydride by the tetraacetylation technique (table XI) After each "acetylation" the catecholamine tetraacetate were isolated by TLC chromatography and between "acetylations" they were eluted in chloroform and washed repeatedly with water to remove any small amounts of catecholamines which might have formed from catecholamine acetates destroyed during the elution procedure and which could have led to underestimation of the ratio Although some samples showed a slight decrease in the ratios which might indicate that a small exchange of acetyl groups really had taken place others instead showed increased ratios after the

Table IX

Distribution of ^{14}C-activity in silica gel chromatograms (from starting line to front) following second acetylation of ^{14}C-adrenaline triacetate. Chromatography of the chloroform extract in system S_7. The ^{14}C-activities are given in per cent of the total activity of the chromatogram

	Fractions in cm from starting line								
	0-2	2-4	4-6	6-8	8-9	9 10	10-11	11 12	12 14
						A-triac		A-tetraac	
% ^{14}C-activity	0.28	0 00	0.06	0.28	0 09	1 76	0.31	96 73	0 49

Table X

^{3}H/^{14}C ratios in successive chromatographies of ^{14}C-noradrenaline and ^{14}C-adrenaline which had been triacetylated with acetic-^{3}H anhydride and after the fourth chromatography were acetylated further to tetraacetate with unlabelled acetic anhydride

Catecholamine triacetate	^{3}H/^{14}C ratios after chromatography no 1	2	3	4	5	6
Noradrenaline	6.51	5.50	5 74	5.59	5.92	
Noradrenaline	73.5	73.0	75.8	71.3	70.6	70.2
Adrenaline	9.36	5 78	5.85	5.96	5.81	5.85
Adrenaline	37 7	13 1	12.8	12.7	12.2	

Table XI

^{3}H/^{14}C ratios obtained for doubly labelled catecholamine triacetates which were acetylated repeatedly with 20 µl unlabelled acetic anhydride and 100 µl pyridine for 2 hours at 60 C. Isolation took place by TLC chromatography between each "acetylation after which aliquots were taken for determination of the ^{3}H/^{14}C ratios by liquid scintillation counting.

Catecholamine acetate	Stable ^{3}H/^{14}C ratio for triacetate	Ratio after repeated tetraacetylation procedures in per cent of the triacetate ratio: Real tetra-acetylation	Repeat procedure 1	2	3
Adrenaline	7 73	90.8	93.9	88 4	89 1
Adrenaline	8.82	99.5	96.5	92 4	88 7
Adrenaline	7.21	105.8	101 9	103.9	105.4
Adrenaline	11 40	90 4	84.9	78.9	80 1
Adrenaline	7.61	98.9	92.6	92.5	85.5
Adrenaline	7.30	101.6	95.6	97.0	
Noradrenaline	2.09	96 7	114.8	113.9	
Noradrenaline	1.23	99.2	96 7	96 7	94.3
Mean		97.9	97 1	95.5	
± SEM		1.8	3.0	3 7	

"acetylations" The reason for the raised ratios is not clear However the mean values did not show any significant effect on the ratios of the repeated "tetraacetylations" Therefore although slight variations in the ratio may occur the method should nevertheless be of value as a check of purification Whether a large or small excess of acetic anhydride was used at the second acetylation had no influence on the result neither had minor variations of temperature or time

Second acetylation thus gives a possibility of changing the chromatographic properties of catecholamines with one hydroxyl group on the β-carbon such as noradrenaline adrenaline and isoprenaline

2. Silylation

Another possible method of preparing a new derivative with altered chromatographic properties from catecholamine triacetate by utilizing a free OH group on the β-carbon atom is silylation Brochmann-Hanssen and Bærheim Svendsen (1962) showed that triacetylated catecholamines can be silylated by treatment with hexamethyldisilazan in tetrahydrofurane which gives more stable derivatives for gas chromatography than the pure triacetates. With this method they obtained a gas-chromatographically uniform product The authors suggest that a trimethylsilyl ether is obtained from the free alcoholic hydroxyl group in adrenaline or noradrenaline triacetate This interpretation is supported by our own experiments with this technique in which doubly labelled adrenaline and noradrenaline triacetates were silylated after repeated chromatographic purification Essentially uniform products were obtained with less polar properties than triacetate but with retention of the $^{3}H/^{14}C$ ratios.

Horning, Moss and Horning (1967) used *N O-bis-(trimethylsilyl)-acetamide* (Tri Sil/BSA) for silylation of non-acetylated catecholamines When we applied their technique to acetylated catecholamines it gave a considerably more complete reaction than the method described above Silylation with 0.2 ml *Tri Sil/BSA* was performed after freeze-drying of catecholamine triacetates. The reaction time was 15 min at 60°C The reaction was interrupted by freeze-drying of the mixture after which the silylated catecholamine triacetates were dissolved in chloroform and chromatographed two-dimension ally on freshly activated silica gel TLC acetone chloroform (4 1) (S_{11}) Distinct spots on the chromatograms, closer to the front than the catecholamine triacetates and well apart from these were obtained

Table XII presents the results of silylation of a batch of ^{14}C-adrenaline ^{3}H-triacetate which after chromatography to constant $^{3}H/^{14}C$ ratio was

Table XII
$^{3}H/^{14}C$ ratios of ^{14}C-adrenaline ^{3}H-triacetate after silylation. The triacetate was purified to a constant ratio and divided into three samples which were silylated with 0._ ml Tri Sil/BSA at 60 C samples 1 and 2 for 15 min, sample 3 for 60 min. Isolation took place by two-dimensional TLC in system S_{11} in both directions. After elution with chloroform the $^{3}H/^{14}C$ ratios were determined in aliquots and chromatography was then repeated in th same system in both directions.

Sample	$^{3}H/^{14}C$ ratio for A-triacetate	$^{3}H/^{14}C$ ratio after silylation and chromatography 1 2	$^{3}H/^{14}C$ ratio after chromatography 3 4
1	12 16	11 76	12.26
2	12 16	11.94	1 16
3	12.16	11.89	12 10

divided into three samples which were silylated separately The $^{3}H/^{14}C$ ratio remained essentially constant after the silylation indicating that only the free OH group on the β-carbon atom was silylated No exchange between the silyl and acetyl groups thus seemed to take place in the reaction In other experiments catecholamine tetraacetates, i.e completely acetylated catechol amines, were treated with Tri Sil/BSA as above no change in the Rf values nor in the $^{3}H/^{14}C$ ratio occurred This supports the view that no destruction of the acetates takes place in association with silylation with our technique In the experiments in table XII only ? 3% of ^{14}C-adrenaline ^{3}H-triacetate escaped silylation. A reaction time of 15 min seems to be fully adequate for a good recovery Like other silyl derivatives the silylated catecholamine acetates are sensitive to humidity and they should therefore be eluted from the chromatograms with and stored in chloroform

As a control that a double isotope labelled catecholamine triacetate which has been purified to constant $^{3}H/^{14}C$ ratios does not despite purification contain residual ^{3}H-impurities, silylation thus seems to be a useful method if a free hydroxyl group is present

Other techniques for changing the chromatographic properties

In an attempt to find a method for second derivation of catecholamine triacetates containing no free hydroxyl group e.g dopamine triacetate other techniques for derivation were studied One possibility was selective hydrolysis of acetyl groups, which would give a mono- or diacetate whereby the chromatographic properties would be changed and the $^{3}H/^{14}C$ ratios would fall to 1/2 or 1/3 of the original ratio of the catecholamine triacetate Such a

technique has been employed by Kliman and Peterson (1960) who converted aldosterone diacetate to more polar monoacetate Our attempt to selectively split the acetyl groups on the benzene ring by acid or alkali gave no homogenous product but a mixture of mainly mono- and diacetate However two techniques to achieve such splitting with retention of the amino acetyl group have been described Goldstein *et al.* (1960) prepared N-acetyl-3 metoxynoradrenaline from the diacetate by deacetylation with hog kidney acylase In another experiment Goldstein and Musacchio (1962) used N-acetyldopamine prepared by hydrogenolysis of dopamine triacetate with $LiAlH_4$ Both techniques seem to be of great interest

ISOTOPE FRACTIONATION

An accepted index of radiochemical purity within a spot on a chromatogram is the finding of a constant $^{3}H/^{14}C$ ratio in successive fractions of the spot (Keston and Udenfriend 1949 Kliman and Peterson 1960) In our experiments with doubly labelled catecholamine triacetates purified by repeated chromatography until constant isotope ratios were attained when the spot was taken as a whole we were surprised to find that the ratio varied within the spot. The phenomenon occurred whether the triacetates were derived from tissue extracts or from acetylation of pure catecholamines

In almost all spots studied by separate measurement of the radioactivity in fractions of the spot some displacement was seen between the ^{3}H activity peak and the ^{14}C peak The ^{14}C activity seemed to move a little faster In silica gel TLC this phenomenon was fairly insignificant (see fig 4) but in paper

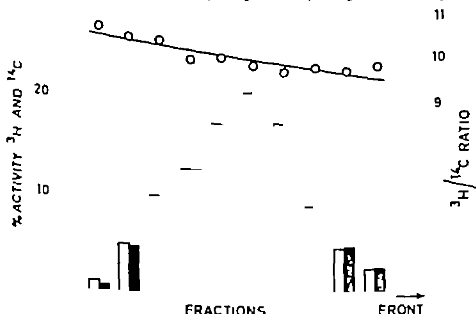


Fig. 4 *Run 201* chromatography of ^{14}C-adrenaline ^{3}H-triacetate on silica gel in system S_2 The intraspot distribution of ^{3}H-activity (black bars) and ^{14}C-activity (white bars) in successive 0.2 cm fractions are shown in the histogram and on the left ordinate The $^{3}H/^{14}C$ ratios for the corresponding fractions are shown at the top (ratios on log scale) and read on the right-hand ordinate The straight line has been fitted by the least-squares method discarding the extreme points at either end

chromatography it was much more important For instance in a paper chromatography of ^{14}C-adrenaline ^{3}H triacetate in toluene ethyl methyl ketone (3 1) for 15 hours the $^{3}H/^{14}C$ ratio in the fraction nearest the leading edge of the spot was about 35% lower than that in the fraction nearest the *trailing edge (fig 5) As seen in these and several following figures the* $^{3}H/^{14}C$ ratio within the spots decreases continuously from the trailing towards the leading edges This variation in the isotope ratios could conceivably be caused by a radioactive impurity in the catecholamine triacetate migrating with an Rf value only slightly different from those of the catecholamine triacetates in the different chromatographic systems. Such an impurity would naturally lead to false results in quantitative determinations with our technique In our method the phenomenon was observed both for adrenaline and noradrenaline triacetate especially in system P_4 and P_6

Now a shifting intraspot ratio need not necessarily imply that impurities are present it may be due to isotope fractionation to different Rf values for chemically identical but isotopically different *molecules As a matter of fact a*

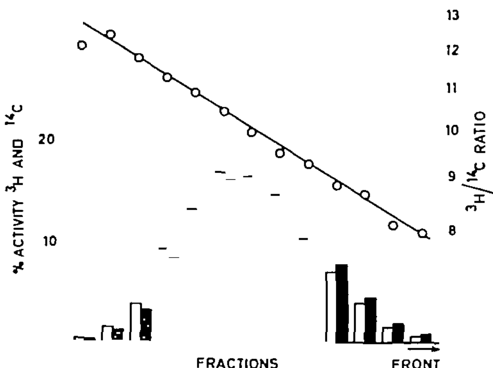


Fig. 5 *Run 204* chromatography of ^{14}C-adrenaline ^{3}H-triacetate on paper in toluene ethyl methyl *ketone (3 1) for 15 h descending system The intraspot distribution* of ^{3}H activity (black bars) and ^{14}C-activity (white bars) and the $^{3}H/^{14}C$ ratios in successive 0.5 cm fractions are shown. Further explanations see fig. 4

shifting intraspot ratio where the ratios plotted on a semilog grid fall on a straight line (see fig 4-8) strongly indicates isotope fractionation According to Dr Klein (Klein 1971 personal communication) there must be a *very large* impurity component to produce displacement with an approximately linear isotope ratio plot Small impurities simply produce an upturn or downturn in an otherwise linear plot

Klein in his comprehensive review (1966) points out that isotope fractionation first reported in the mid 1950 s (Piez and Eagle 1955 1956 Speeding Powell and Svec 1955) is considerably more common than was previously realized Klein and coworkers (Klein Simborg and Szczepanik 1964 Liragh Sealey and Klein 1965) have shown that isotope fractionation occurs in chromatography of doubly labelled cholesterol acetate as well as aldosterone diacetate Isotope fractionation has also been shown for several other radioactively labelled substances (see review by Klein 1966)

Chromatographic evidence of isotope fractionation

Different techniques have been employed in attempts to find out whether a variation of the isotope ratio within a spot is due to an impurity or only to the occurrence of isotope fractionation of an otherwise homogenous substance Brown *et al.* (1956) rechromatographed their substances repeatedly in the same system but cut the spot symmetrically around the centre after each run so that only the middle 2/4 was rechromatographed each time Prior to each further chromatography recrystallized carrier substance was added If the variation in the intraspot ratio remains constant after each chromatography isotope fractionation probably is the only cause of the intraspot variation since any impurity would become diluted by the procedure

We studied ^{14}C-adrenaline ^{3}H-triacetate by Brown s technique (see fig 6) The adrenaline triacetate was first purified chromatographically until constant $^{3}H/^{14}C$ ratios (9.97) were obtained in the eluates This was followed by three successive paper-chromatographies in toluene ethyl methyl ketone (3 1) for 15 hours each discarding 1/4 of the spot at either end according to Brown *et al.* The radioactivity was measured in a strip of the spot after each chromatography in 0.5 cm wide fractions from the trailing to the leading edge The isotope ratios of the entire spots remained constant (chromatography I 9.99 II 9.95 III 10.00) As seen in fig. 6 a progressively decreasing ratio was obtained within the spots from the trailing to the leading edges after all chromatographies, and the intraspot change in isotope ratio was of the same order of magnitude after each chromatography strongly supporting that isotope fractionation had played a part. In the first chromatography (run 202)

in fig. 7 fairly similar intraspot changes in the isotope ratio were obtained in the three samples after the rechromatography This experiment also provides strong evidence of isotope fractionation and contradicts the possibility of a large impurity as the cause of the variation. However the small upturn at the right end of the rear spot (run 303) probably is due to a small impurity

Further attempts were made to support the occurrence of isotope fractionation Fig 8 shows that a change of the intraspot ratio occurs also for adrenaline tetraacetate (^{14}C-adrenaline (β)-^{1}H-acetate (3 4 N)-^{3}H-triacetate) purified by paper chromatography in system P_4 for 4 hours In this experiment triacetylation was performed in the conventional way and followed by purification until constant ratios. Then further acetylation to the tetraacetate with unlabelled acetic anhydride was made If the changes in the intraspot ratios in the triacetate spot had been caused by an impurity a second acetylation with alteration of the chromatographic properties of the adrenaline acetate should have removed or greatly reduced the impurity There would then no longer be the same pronounced change in the isotope ratio in the tetraacetate spot As seen in the figure however there still was a significant intraspot variation in the same direction as with triacetate However as the

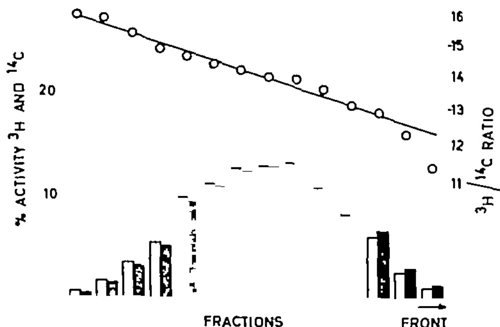


Fig. 8. *Run 401* chromatography of ^{14}C adrenaline (β)-^{1}H-acetate (3 4,N)-^{3}H-triacetate on paper in system P for 4 h descending system The intraspot distributions of ^{3}H-activity (black bars) and ^{14}C-activity (white bars) and the ^{3}H/^{14}C ratios in successive *0.5 cm fractions are shown. Further explanations see fig. 4*

ratios in the figure do not seem to be randomly distributed around the straight line it cannot be excluded that besides isotope fractionation some small impurity could have been present in the tetraacetate spot.

Mass spectrometry

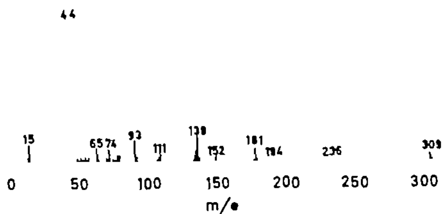
If heavy radioactive contamination of the mass spectrometer had been acceptable it might have been possible directly to detect differences in the isotopic composition of the ion-species between the leading and the trailing edge of the spot However such a degree of contamination was not permissible Therefore mass spectrometry was only used to exclude the presence of an impurity with Rf values very similar to the triacetate in all systems used which might explain the shifting intraspot ratio

An experiment was performed where 100 μg adrenaline was triacetylated with acetic anhydride and a small amount of ^{14}C-adrenaline with acetic ^{3}H anhydride The two samples were mixed and chromatographed together on silica gel to constancy of the $^{3}H/^{14}C$ ratio The sample was then chromatographed on paper in system P_4 The fastest and slowest 1/3 of the spot were eluted The ratio in the fastest segment was 9 19 and that in the slowest segment 10.54 The mean ratio in the entire spot was 9 74 The two eluted fractions were once rechromatographed on silica gel in system S_3 This did not change the ratios The two fractions were then studied by mass spectrometry without any further derivative formation

The mass spectra at 80°C 70eV were identical with each other and with that of crystallized adrenaline triacetate shown in fig 9 No signs of any impurities could be detected, neither in the sample from the rear nor in that from the front of the paper chromatography spot despite the differences in the $^{3}H/^{14}C$ ratios.

A foreign product which could not be identified with certainty was seen in the form of several unknown fragments in the mass spectrum in both samples however It was ionized *in vacuo* in the mass spectrometer at 40 50°C while adrenaline triacetate was ionized at 70 80°C This product probably arose from the paper since it could be greatly reduced by washing the paper for 24 hours in the chromatography solvents prior to the chromatography Further the product was not detected in the original adrenaline triacetate before paper chromatography

In order to find out whether the unknown substance could affect the $^{3}H/^{14}C$ ratio i.e. whether it might possibly be a radioactively labelled impurity one of the samples was introduced into the mass spectrometer and was allowed to become ionized at 50 C until the ionic flow at this temperature



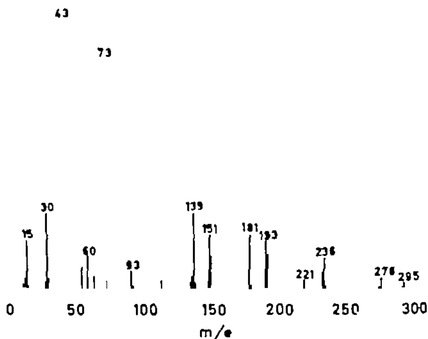


Fig. 9 Mass spectrum of adrenaline triacetate (mol. wt. 309) direct inlet 70 eV 80°C (above) and of noradrenaline triacetate (mol. wt. 295) direct inlet, 70 eV 100 C. Mass spectrometer Varian CH7

had practically ceased as a sign that the substance had left the sample The sample was then taken out and dissolved in methanol and the $^3H/^{14}C$ ratio determined Before the experiment the ratio for th adrenaline triacetate in the sampl was 9 19 and after the experiment 9 15 demonstrating that the substance that had left the sample had not contained ^3H or ^{14}C that could have given rise to the intraspot variation in $^3H/^{14}C$ ratio

Klein analysis of the data

Klein and coworkers (Klein Simborg and Szczepanik 1964 Laragh Sealey and Klein 1965 Klein 1966) have recommended two different kinds of criteria for ruling out anything but isotope fractionation as explanation of the shifting intraspot ratios the position of the peaks calculated in a certain manner must not differ by more than 1% and the dispersions of the peaks must no differ by more than 3% (Klein 1970) Dr Klein has very kindly run our data through his computer program His conclusion is that a true isotope fractionation was present in all chromatograms studied (Klein 1971 personal communication). Table XIII shows the results of his calculations. In all runs the unit of dispersion is a 0.5 cm fraction except in run 201 (silica gel) where 0.2 cm fractions were used Evidently the dispersion of ^{14}C is remarkably similar to that of ^3H within the single run The relative displacement between the ^3H and ^{14}C peaks (% Δ M) is below 1% everywhere The displacement has been calculated by different procedures, the straight forward probit analysis is the most sensitive to asymmetry in the peaks, which may be the reason for the large standard error in several runs. The standard error incidentally only gives information about the internal consistency of each run and cannot be used for judging the significances in % Δ M *between* runs.

Practical consequences of isotope fractionation

Isotope fractionation of the order of magnitude shown to occur in the paper-chromatographic system used above can lead to grossly false results in the determination of catecholamines with double isotope techniques if the whole spot is not taken for elution Therefore the spot must be very carefully cut out The technique used (see p 33) identifies the leading and the trailing edge of the spot with fairly good precision However as we usually cut a narrow strip in the centre of the chromatograms we may miss asymmetrical or irregular spots. This may of course give Fortunately if only a small peripheral part of a spot is missed the total $^3H/^{14}C$ ratio is small Although the ratio of the

Table XIII
Klein analysis of the chromatograms shown in fig. 4 8 The unit of dispersion and displacement is fractions. In run 201 each fraction was 0.2 cm in the other runs 0.5 cm. The displacement between the ^{3}H and ^{14}C peaks is in per cent of the distance (in fractions) between the ^{3}H peak and the front. The isotope-ratio analysis I is calculated without and II with dispersion corrections.

Run and fig	Dispersion ± SD		Displacement ΔM %		
	^{3}H	^{14}C	Probit analysis	Isotope-ratio analysis I	Isotope-ratio analysis II
201/3	1.9877 ±0.0331	1.9999 ±0 0344	0 1856 ±0.2156	0 1520 ±0.0360	0 1455 ±0 0244
202/5	2.6586 ±0.0847	2.6408 ±0.0825	0 4204 ±0.2344	0 4966 ±0.0321	0 4896 ±0.0401
203/5	2.9135 ±0.0381	2.9048 ±0.0382	0 4308 ±0 1077	0 4540 ±0.0139	0 4488 ±0 0130
204/5	2.0406 ±0.0222	2.0380 ±0 0229	0.3664 ±0.0628	0.3764 ±0.0150	0.3773 ±0 0136
301/6	2.2148 ±0 1181	2.2001 ±0 1151	0 4101 ±0.3150	0.3857 ±0 0389	0.3674 ±0.0459
302/6	2.5874 ±0 0321	2.5726 ±0.0417	0 4202 ±0 1061	0.5099 ±0.0198	0.5003 ±0 0240
303/6	2.6277 ±0.0383	2.6116 ±0.0402	0 4439 ±0 1114	0.5077 ±0.0163	0 4984 ±0.0205
401/7	2.8032 ±0.0409	2 7895 ±0.0407	0 8102 ±0 2700	0.8986 ±0.0745	0.8954 ±0.0580

different from that of the total spot, the radioactivity at the edges of the spot is very low compared to that in the centre After a spot has been cut out we always check (by staining or radioactive counting) the areas immediately above and below to exclude that any appreciable parts have been left

If the whole spot is not to be eluted for example when an impurity with a similar Rf value is to be avoided the spot must be cut symmetrically around the midpoint of the radioactive peak The midpoint should preferably be established by direct measurement of the radioactivity from a narrow strip of the spot or by radioactive scanning. In order to establish the centre of the peak in such analyses the spot should be divided into at least 8 segments (Laragh Sealey and Klein 1965). If spots are cut however it must be observed that there is a risk of getting false ratios if the spots happen to be very irregular

Klein and Szczepanik (1969) have shown in their studies of doubly-labelled amino acids that several secondary phenomena affect isotope fractionation For instance on the addition of tert.-butanol to certain chromatographic systems the separation between the ^{3}H and ^{14}C peaks is reduced This may conceivably

be due to the capacity of tert.-butanol to break hydrogen bonds and local solvent structures. Following them we found that the isotope fractionation could be reduced if toluene ethyl methyl ketone tert.-butanol (3:1:1) was used instead of toluene ethyl methyl ketone (3 1) for chromatography of adrenaline triacetate. However the system containing tert.-butanol showed more tailing. It was found further that almost no isotope fractionation occurred in a paper-chromatographic system containing tert.-butanol described by Laverty and Sharman (1965) (petroleum ether tert.-butanol water 8 3 5). Unfortunately this chromatographic system is not particularly efficient, the migration of the substances is very slow and different catecholamine acetates are separated rather poorly from each other

Conclusions concerning criteria for purity and identity of the triacetate in the double-isotope method

Different criteria can be used for making the judgement that a compound is pure and identical with a reference sample. With regard to the triacetates under discussion these may now be summarized as follows

A. Criteria for purity

1. Constancy of the isotope ratios on repeated chromatography in different systems
2. Absence of radioactive contaminants in the areas on the chromatography plate immediately above and below the triacetate spot
3. The variation in the intraspot ratio after paper chromatography shows a constantly changing ratio due to isotope fractionation without signs of interference from any second substance
4. Klein analyses shows small enough separation between the peaks and similar enough dispersions.
5. Second acetylation does not change the isotope ratio
6. The ratio remains unchanged after silylation
7. The ratio remains unchanged after crystallization

B Criteria for identity

1. Expected Rf values at chromatography in 5 6 different systems
2. The Rf values after second acetylation agree with those of the tetraacetate of the catecholamine in question
3. The Rf values after silylation agree with those of the silyl derivative of the triacetate of the catecholamine in question

Chapter V

QUANTITATIVE DETERMINATIONS BY THE TRIACETATE TECHNIQUE

The quantitative determinations of adrenaline and noradrenaline presented in this chapter (table XIV XX) have been performed with several different batches of acetic ^{3}H anhydride and tracer ^{14}C-catecholamines The specific activity of the acetic ^{3}H anhydride was around 400 Ci/mol in the experiments shown in table XVI and XVIII XX and in the other experiments around 100 Ci/mol. Since the specific activity of the ^{14}C-catecholamines was 20 60 Ci/mol the specific activity of 400 Ci/mol was unnecessarily high (see p 38)

Two standards were used in each analytical run (see p 14) In most of the experiments 300 000 cpm of the tracer catecholamines and 10 μg of each unlabelled catecholamine were used as standard I and 300 000 cpm of each tracer as standard II We now recommend somewhat lower amounts of tracers and unlabelled catecholamines in standard I However when these experiments were performed we did not yet realize the importance in critical experiments of adjusting the $^{3}H/^{14}C$ ratio of standard I to the predicted isotope ratios of the samples Therefore the random errors might be higher than necessary in experiments where the amount of unknown in samples was less than 0 1 μg.

Precision and accuracy of the method

In order to test the precision and accuracy of the method 0.01 10 μg of adrenaline bitartrate or noradrenaline bitartrate was added to 200 μl portions of 1 mM HCl ^{14}C-adrenaline or ^{14}C-noradrenaline were added as tracers, see table XIV and XV In some experiments the tracers had been supplied dissolved in acetic acid These were purified over alumina immediately before use and eluted with 0 1 N formic acid In other experiments freeze-dried catecholamine bitartrate was used as tracer This was dissolved in 0 1 N formic acid immediately before use Acetylation was performed in the usual way with 7 μl acetic anhydride solution (acetic ^{3}H anhydride-benzene 1:4) After extraction in chloroform the catecholamine triacetates formed were purified by chromatography until a constant $^{3}H/^{14}C$ ratio was obtained The amount of catecholamine in the samples was calculated from the mean value of the ratios in the last 2 4 chromatographies, depending on when an essentially constant ratio had been reached

Table XIV shows the values obtained in the determination of adrenaline On analysis of 1.0 μg in 5 samples the accuracy was reflected by a mean value ± SD

Table XIV

Determination of known amounts of adrenaline (A) in aqueous solutions with the triacetate double isotope technique. Italicized ratios were utilized in the calculations.

Unlabelled A	Tracer ^{14}C-A	$^{3}H/^{14}C$ ratio after chromatography						Mean ratio	Unlabelled A recovered	
μg	μg	S_1	S_2 S_3	P_4	S_5	P_6	S_7		μg	$\bar{x} \pm SD$
10.0	0 79	19.8	18.3	*16.5*	*18.6*	*19.2*		18.09	9.96	
1.0	0.33	31 6	25.6	*22.9*	*25 1*	*77.3*	*26.0*	25.32	0 97	
1.0	0.33	31.0	26.0	*24.8*	*26.8*	*26.9*	*27.3*	26 46	1.03	
1 0	0.33	30 4	26 4	*25 1*	*26.7*	*27 0*	*27 6*	26.60	1.04	1.010 ± 0.028
1.0	0.33	29.5	26.6	25 4	26.7	*26.5*	*26.2*	26 17	1.02	
1 0	0.33	29.8	28.0	*23.0*	*7 1*	*26.3*	*76.2*	25.67	0 99	
0.1	0.33	11.0	9 1	*8 0*	*8.3*	*8 9*	*8 6*	8 45	0 108	
0 1	0.33	10.2	8.8	*7.8*	*8.2*	*8.7*	*8 6*	8.34	0.103	
0 1	0.33	10.5	8.6	*8 0*	*8.0*	*8.3*	*8.5*	8 19	0.095	0 103 ± 0.007
0 1	0.33	11.5	8.9	*8 4*	*8.5*	*8 9*	*8.5*	8.52	0 112	
0 1	0.33	11 1	8.6	*7.8*	*8 1*	*8.4*	*8 7*	8.25	0 098	
0.01	0.008	37.5		*3 7*	*3.5*	*3.3*		3.50	0.0108	
0.01	0.008	44.3		*3 1*	*3 6*	*3 7*		3 44	0 0106	0.0107
0.01	0.008	40.2	–	*3 4*	*3.5*	*3.5*		3 44	0 0106	
0.005	0.007	33 4	23 1	7.9	*4 4*	*4 9*		4.66	0.0044	

Table XV
Determination of known amount of noradrenaline (NA) in aqueous solutions with the triacetate double isotope technique. Italicized ratios were utilized in the calculations.

Unlabelled NA	Tracer ^{14}C-NA	$^{3}H/^{14}C$ ratio after chromatography						Mean ratio	Unlabelled NA recovered	
μg	μg	S_1	S_2 S_3	P_4	S_5	P_6	S_7		μg	$\bar{x} \pm SD$
10.0	0.84	84.5	*81 0*	*80 9*	*81.5*	–	*81.2*	81 10	10.26	
1.0	0.20	102.6	21.6	*18.0*	*18.3*			18 18	1.05	
1 0	0.20	56.2	21 4	20 7	*17 9*	*17 9*		17 92	1.03	
1.0	0.20	50 7	25 4	20.6	*17.5*	*19 0*		18.26	1.06	1.058 ± 0.020
1.0	0.20	76.5	22.0	23.8	*18.9*	*18 7*		18 79	1.09	
1 0	0.20	100 4	38.2	30.8	*18.8*	*17 7*		18.22	1.06	
0 1	0 058	–	6.5	4 1	*3 9*	*3 7*	*3 9*	3.83	0 102	
0 1	0 058	–	8.0	4 1	*3.9*	*3 6*	*3 7*	3 70	0.097	
0.1	0 058	–	9.0	4.6	*3 9*	*3.5*	*3 7*	3.68	0.096	0 100 ± 0.003
0.1	0.058	–	7.9	5.2	*4.2*	*3 7*	*3 6*	3.84	0 102	
0.1	0 058	–	6.9	5 1	*4 1*	*3.3*	*4 1*	3.84	0 102	
0.01	0.007	–	16.2	9.9	*6 6*	*7 0*		6 78	0.0099	
0.01	0 007	–	24.0	10 0	*7 4*	*6.9*		7 16	0.0109	0.0106
0 01	0.007	–	19 4	8 7	*7 1*	*7 4*		7.21	0.0109	

Table XVI

Determinations of known amounts of adrenaline (A) and noradrenaline (NA) in aqueous solutions with the triacetate double isotope technique. 1 and 3 adrenaline to noradrenaline 1/100 2 and 4 adrenaline control 5 noradrenaline to adrenaline 1/100 6 noradrenaline control 7 only [1] C-noradrenaline tracer together with 10 μg adrenaline and 1.3 μg ^{14}C-adrenaline 8 ^{14}C-noradrenaline tracer 9 ^{14}C-adrenaline tracer Italicized ratios were utilized in the calculations. CA catecholamine.

Sample	Unlabelled CA subst.	μg	Tracer ^{14}C-CA μg	$^{3}H/^{14}C$ ratio after chromatography S_1	S_2 S_3	P_4	S_5	P_6	S_7	Mean ratio	Unlabelled CA recovered μg
1	A	0.1	0.066	28.3	*19 7*	*21 7*	*21.3*	*21 1*		21.0	0 112
	NA	10	1 1	79 1	*72 4*	*77.3*	*75.8*	*71 1*		74 1	10.0
2	A	0 1	0.066	32 7	*20.0*	*[illegible]0.4*	*20.2*	*18.6*	*1.3*	20 1	0 104
3	A	0.01	0.0066	97.3	*23.3*	*23 0*	*21 1*			22.5	0.0124
	NA	1.0	0.11	77.6	*75.5*	*74.5*	*75.9*	*74 1*		75.0	1 01
4	A	0.01	0.0066	307	*23.3*	*[illegible]3 1*	*19 6*	*20.9*		21 9	0 0118
5	A	10	1.3	*65.5*	*65.2*	*66 9*				65.9	10 0
	NA	0 1	0 097	29.2	*15 9*	*15 7*	*15 7*	*15.3*	*15 7*	15 7	0 111
6	NA	0 1	0.097	32.1	16.9	*15.8*	*16 0*	*15 7*	*15 7*	15.8	0.112
7	A	10	1.3	*65 1*	*64.8*	*67.8*				65.9	10.0
	NA	–	0.0097	237	–	*7 7*	*7 9*			7.84	0 0007
8	NA	–	0.097	19.6	*7.2*	*7.3*	*7.3*	*7 7*	*7 1*	7.32	
9	A	–	0 066	15.9	*8.0*	*8.1*	*8.0*	*7.3*	*7 6*	7.80	

of 1.010 ± 0.028 μg The coefficient of variation V was 2.8% An analysis of 5 samples with 0 1 μg gave a mean value of 0 103 ± 0.007 μg and V 6.8% An analysis of 3 samples with 0.01 μg gave a mean value of 0 0107 μg range 0.0106 0.0108

In the determinations of 1.0 μg of adrenaline the ratio between the catecholamine to be analysed and the amount of tracer added was 3/1 in the determinations of 0 1 μg it was 1/3 The higher coefficient of variation in the later experiments may be due to the relatively large amount of tracer but the results still show that relatively good precision can be obtained even in analyses where owing to the very small amount of catecholamine in the sample the amount of tracer added is 2 3 times higher than the amount of catecholamine to be determined

Table XV gives the values obtained in determinations of known amounts of noradrenaline An analysis of 5 samples with 1.0 μg gave a mean value ± SD of 1 058 ± 0.020 μg, V 2.0% Five samples with 0 1 μg gave 0 100 ± 0.003 μg V 3.0% The mean value for 3 samples of 0.01 μg was 0.0106 μg range 0.0099 0.0109

The overall recovery of the added tracer varied between 5% and 20%. The variation was mainly due to the fact that the amount taken for determination of the ratio after each chromatography depended upon the activity of the sample More was taken from weak samples

One of the greatest problems in quantitative determinations by the fluorometric techniques is the difficulty of assaying one catecholamine in the presence of a very large excess of another in the same tissue or solution One of the principal aims of our study was to develop a technique allowing determinations in this situation Table XVI illustrates some experiments in which adrenaline and noradrenaline of greatly different concentrations were assayed in aqueous solutions In experiment 1 and 3 adrenaline was assayed in the presence of a 100 times higher concentration of noradrenaline In experiments 2 and 4 the same amount of adrenaline was assayed without the presence of noradrenaline The amount of adrenaline obtained in experiment 1 was 0 112 μg as against 0 104 μg in the control analysis (experiment 2) In experiment 3 the corresponding value was 0.0124 μg as against 0.0118 μg in the control (experiment 4) In experiment 5 noradrenaline was assayed in the presence of a 100 times higher concentration of adrenaline Here the solution was found to contain 0 111 μg noradrenaline compared with 0 112 μg in the control analysis (experiment 6) without the presence of adrenaline In experiment 7 the reagent blank for noradrenaline was determined in the presence of a large amount of adrenaline An apparent amount of unlabelled noradrenaline of 0.0007 μg was found This corresponds to less than 1 part

noradrenaline in 10 000 of adrenaline an amount which could have been present despite recrystallization

Even though some of the values are slightly high the experiments illustrate that the method can be used with fairly good accuracy in analyses where very large concentration differences between two or more catecholamines can be expected

Tissue experiments

Up to now the method has been mainly applied to the problems of adrenaline in brain tissue This work is still in progress but some results will be presented here to demonstrate the applicability of the method

In order to study the precision and accuracy in determinations in brain tissue recovery experiments were performed Four untreated male Sprague Dawley rats with a body weight of 350 450 grams were stunned by a blow on the head The brains were removed immediately and homogenized together in 0 4 N perchloric acid The extract was treated as described under "Methods" except that no tracer was added primarily Immediately before isolation over alumina the extract was divided into 8 equal samples which were analysed separately Each sample thus contained endogenous catecholamines correspond ing to one half of a brain. To samples 1 4 were added 0.5 μg each of adrenaline and noradrenaline ^{14}C-adrenaline and ^{14}C-noradrenaline were added as tracers to all 8 samples. After isolation over alumina the catecholamines were acetylated in the usual way with acetic ^{3}H anhydride after which chromatographic purification was performed until constant ratios were attained (table XVII) Values for noradrenaline of 0 747 ± 0.015 μg (mean ± SEM) were obtained for samples 1 4 and 0.248 ± 0.008 μg for the controls (samples 5 8) Subtraction of the amount of endogenous noradrenaline (mean value for samples 5 8) gave an added amount of 0 496 ± 0.017 μg of noradrenaline in samples 1 4 as compared with an expected 0.5 μg The amount of adrenaline found in samples 1 4 was 0.525 ± 0.023 μg (mean ± SEM) thus there may be a little endogenous adrenaline in the samples (see below). However in samples 5 8 where only 1600 cpm ^{14}C-adrenaline was used as tracer the recoveries were too low in this analytical run to permit chromatographic isolation until constant ratios were obtained The amount of endogenous adrenaline in the brain could therefore not be calculated with certainty in these experiments.

Tables XVIII and XIX present the results of determinations of the concentrations of noradrenaline and adrenaline in the brain of another group of untreated male Sprague Dawley rats with a body weight of 300 500 grams.

Table XVII

Recovery of noradrenaline (NA) and adrenaline (A) from perchloric acid extracts of rat brain. The extracts from 4 brains (weights 1.23 1.21 1 19 1 13 gram) were carefully mixed and divided into 8 equal samples. To samples 1 4 were added 0.5 μg each of noradrenaline and adrenaline Italicized ratios were utilized in the calculations. CA catecholamine

Sample	Added CA subst	Added CA μg	Tracer ^{14}C-CA μg	$^3H/^{14}C$ ratio after chromatography S_1	S_2 S_3	P_4	S_5	P_6	Mean ratio	Unlabelled CA found μg	Unlabelled CA found $\bar{x} \pm SD$
1	NA	0.5	0.12	–	20.8	*1.2*	*23 0*	*[illegible]3*	*22 17*	*0 749*	
2	NA	0.5	0 12	–	23.5	*21 0*	*22.1*	*22.0*	21.69	0 734	0 744 ± 0.030
3	NA	0.5	0 12	–	22.8	*20 7*	*21.2*	*21 4*	21 08	0 710	
4	NA	0 5	0 12	–	26.3	*22.4*	*23.2*	*23 0*	22.87	0 781	
5	NA	–	0 12	–	10 7	*9 1*	*8.9*	*9 4*	9 15	0.239	
6	NA	–	*0 12*	–	*10.2*	*9.5*	*9 1*	*9 9*	9 48	0.255	0.248 ± 0 016
7	NA	–	0 12	–	*8.8*	*9 0*	*8 7*	*8.9*	8.86	0.231	
8	NA	–	0 12	–	10.3	*9 8*	*9 6*	*10 1*	9.83	0.266	
1	A	*0.5*	*0.07*	–	–	18.5	*18 7*	*17 0*	17.86	0.549	
2	A	0.5	0 07	–	31 6	18 7	*15 0*	*15.5*	15.25	0 459	0.525 ± 0 045
3	A	*0.5*	0 07	–	34.8	25.2	*18.3*	*17.5*	17 91	0.549	
4	A	0.5	0.07	–	35.3	24 1	*17.3*	*18 0*	17 65	0.542	
5	A	–	0 035	–	97.5	50.2	37.5	*31.5*		(0.050)	
6	A	–	0.035	–	61.5	34 4	30.3	*24 4*		(0.038)	
7	A	–	0.035	–	43.2	–	–	–			
8	A	–	0.035	–	79.9	34.5	27.2	*20 1*		(0.031)	

The animals were stunned by a blow on the head The brains were removed immediately and weighed They were carefully freed from plexus choroideus and all larger branches of a. carotis interna and a basilaris. (In experiment 1 *and 2 however plexus choroideus and several of the main branches of the cerebral arteries were left with the brain*) The brains were then either transferred directly to 0 4 N perchloric acid for analysis or in some cases frozen at −70°C for later analysis One brain was used for each analysis except in experiments 6 7 and 8 9 where brain halves were analysed separately In experiment 10 12 only noradrenaline was determined

The amount of noradrenaline was 0 459 ± 0.050 (SD) μg per gram wet brain (n = 11 each analysis a unit) This value is in good agreement with the amounts reported by several other authors using conventional fluorometric techniques (Bertler and Rosengren 1959 Brodie *et al.* 1960 Callingham and Cass 1963 Moore and Larieviere 1963 Brownlee and Spriggs 1965 Ansell and Beeson 1968) Somewhat lower values 0.2 0.3 μg/g have been obtained by other workers (Montague 1957 Green and Sawyer 1960 Anton and Sayre 1964).

Table XIX shows the results of the adrenaline determinations. The amount of adrenaline was 0.044 ± 0.023 (SD) μg per gram wet brain (n = 9) Especially high values of adrenaline were found in experiment 1 and 2 where the brains were not freed from non-nervous tissue as carefully as in other experiments Experiment 5 showed a surprisingly low value for adrenaline Further work on the determination of adrenaline in the brain aimed at establishing the reason for the large variations in concentration observed in these preliminary analyses, are in progress.

The question of whether adrenaline occurs in the mammalian brain or not has been the subject of much discussion. Older studies in which biological determination techniques were used support the occurrence of adrenaline (Holtz 1950 Vogt 1954) and these results have been confirmed by other investigations on the rat with fluorometric techniques (Montagu 1957 Paasonen and Dews 1958 Gunne 1962 Anton and Sayre 1964) Several other groups have been unable to demonstrate any adrenaline in the brain of the rat (Bertler and Rosengren 1959 Moore and Larieviere 1963 Merrils 1963 Ansell and Beeson 1968) Even if experiments 1 and 2 of table XIX are discarded the remaining ones indicate the presence of a statistically significant amount of adrenaline 0.0335 ± 0 0048 (SEM) μg/g However since some of the ratios in table XIX were still decreasing when further purification had to be stopped the amount of adrenaline found may be slightly overestimated

A small number of preliminary experiments has been performed in order to see if adrenaline and noradrenaline are similarly effected by different interventions. Some of these experiments are presented in table XX. In

Table XVIII

Determination of noradrenaline in rat brain by the triacetate double isotope technique. Mean value ± SD 0 459 ± 0.050 μg per gram wet brain Samples 6 8 half a brain per analysis, the remaining samples, one brain per analysis. In sample 9 (not shown) the analysis was stopped before stable ratios had been obtained Italicized ratios were utilized in the calculations.

Sample	Body wt gram	Tissue wt gram	Tracer ^{14}C-NA μg	$^{3}H/^{14}C$ ratio after chromatography S_1	S_2 S_3	P_4	S_6	S_7	S_2	Mean ratio	Endogenous NA μg/ sample	Endogenous NA μg/ gram
1	430	1.28	0.26	28.0	21.0	*18 4*	*21 4*	*20.9*	*19.8*	20 12	0.60	0 47
2	490	1.31	0.26	26.8	21.6	*20 0*	*20 9*	*19 0*	*20 6*	20 12	0.60	0.46
3	370	1.32	0.27	25.0	*21 0*	*21 4*	*21.3*	–	*22.7*	21.60	0 70	0.53
4	430	1.33	0.26	40.8	23.0	23.0	*21 9*	*19 6*	*20 6*	20 70	0.65	0.49
5	380	1.20	*0.23*	304	–	*17 6*	*17.9*	*17.2*		17.57	0.55	0 46
6	360	0 62	0.27	24	13 7	*11 1*	*12 4*	*12.9*		12 13	0.27	0 44
7		0.64	0.27	18	13.5	*11 9*	*12.8*	*12.5*		12 40	0.30	0 47
8	300	0.63	0 11	52 1	25 0	*24 6*	*24.5*	*27.2*		25 43	0.23	0.37
10	350	1 15	0.26	125	25 4	*19 0*	*21 0*			20 00	0.61	0.53
11	490	1.29	0.26	33.2	*20 1*	*18.5*	*17 0*	*17.3*	*17.5*	17.27	0.50	0.39
12	315	1 15	0.30	51.9	20 1	*19 0*	*18 4*	*19 1*		18 83	0.51	0.44

Table XIX

Determination of adrenaline in rat brain by the *triacetate double isotope technique*. Mean value ± SD 0.044 ± 0.023 μg per gram wet brain. Samples 1–5 1 brain per analysis; samples 6–9 1/2 a brain per analysis. Italicized ratios were utilized in the calculations.

Sample	Body wt. gram	Tissue wt gram	Tracer ^{14}C-A μg	^{3}H/^{14}C ratio after chromatography S_1	S_2 S_3	P_4	S_5	S_7	S_3	Mean ratio	Endogenous A μg/ sample	Endogenous A μg/ gram
1	430	1.28	0.032	346	334	*30.1*	*27.2*	*29.5*	*28.0*	28.83	0.099	0.077
2	490	1.31	0.032	290	[illegible]05	*30.7*	*31.1*	*31.4*	*28.0*	30.30	0.105	0.080
3	370	1.32	0.033	250	–	28.3	*18.3*	*16.9*		17.60	0.056	0.042
4	430	1.33	0.070	190	16[illegible]	*11.0*	*10.6*	*11.5*		11.03	0.039	0.029
5	380	1.20	0.053	75	–	*6.6*	*6.3*			6.47	0.008	0.007
6	360	0.62	0.017	70	17.2	*22.9*	–	*[illegible]3.3*		21.13	0.023	0.037
7		0.64	0.017	100	42.4	*24.7*	–	*22.3*		23.50	0.028	0.044
8	300	0.63	0.011	106	–	*21.5*	*19.2*			20.35	0.022	0.035
9		0.59	0.011	59	–	*21.6*	*1.1*	*2.0*		1.57	0.024	0.041

Table XX

Determination of adrenaline (A) and noradrenaline (NA) in rat brain by the triacetate double isotope technique after various pretreatments. Sample 1 removal of ganglion cervicale superior and adrenalectomy *bilat.* 72 h before death. Sample 2 reserpine 5 mg/kg i.p 24 h before death. Sample 3 Reserpine 5 mg/kg i.p 18 h before death. Sample 4 alpha-methyl-para-tyrosine 500 mg/kg i.p 24 h and bilat. adrenalectomy 96 h before death *Italicized ratios were utilized in the calculations.* CA = catecholamine.

Sample	Body wt gram	Tissue wt. gram	Subst	Added ^{14}C-CA μg	^{3}H/^{14}C ratio after chromatography S_1	S_2 S_3	P_4	S_4	S_7	S_2	Mean ratio	Endogenous CA μg/ sample	Endogenous CA μg/ gram
1	315	1.25	A	0 053	109	–	*6.2*	*7.8*			7.01	0.012	0.010
			NA	0.228	33.0	–	*15 0*	*16.8*			15.9	0 47	0.38
2	270	1.20	A	0.053	57.8	–	*6.3*	*6.4*			6.35	0 006	0 005
			NA	0.228	*33.0*	–	*6 4*	*6 4*	*6 1*		6.29	0.014	0 012
3	450	1.52	A	0.035	–	17.8	*13 0*	*14.2*	*14.3*		13.83	0.033	0.022
			NA	0.035	–	14.6	*13 0*	*13.8*			13 40	0 024	0.016
4	400	1.26	A	0.070	–	11.6	*8.3*	*8.2*	–	*8.5*	8.32	0.014	0 011
			NA	0.070	–	13 7	13.8	*11 9*	*12.5*	*12.6*	12.33	0 059	0.046

experiment 1 bilateral removal of the adrenal glands and the superior cervical ganglion was performed There was still some adrenaline left In experiments 2 and 3 reserpine in a relatively high dose (5 mg/kg) greatly reduced the noradrenaline concentration while adrenaline was less affected (compare with tables XVIII and XIX). Whether it was affected at all is not clear In experiment 4 where an inhibitor of catecholamine synthesis (alpha-methyl-para-tyrosine 500 mg/kg) was given after bilateral adrenalectomy noradrenaline fell but the reduction in adrenaline is not convincing. Thus it seems that the bulk adrenaline does not behave like neuronal noradrenaline However in several of these experiments the amount of added tracer was too large and the results therefore not as reliable as could have been the case with an optimal amount Obviously these preliminary studies do not allow any definite conclusions but further experiments are in progress.

Blanks

In order to be sure that the relatively high values of adrenaline which we noted in the rat brain did in fact correspond to the true concentration, it was important to know the blank values. To obtain a tissue blank is difficult however since almost all tissues contain catecholamines As an extreme case we performed "acetylation" of 200 μl distilled water with 7 μl acetic ^3H anhydride benzene (1:4 vol/vol) specific activity 100 Ci/mol Unlabelled catecholamine triacetate was then added and this was followed by extraction in chloroform and repeated chromatography (table XXI) After five chromatographies 121 cpm ^3H activity was found in the adrenaline triacetate spot and 32 cpm in the noradrenaline triacetate spot (above backgrounds) If the overall recovery in the method were as low as 5% this corresponds to 4 ng apparent adrenaline and 1 ng apparent noradrenaline Two kinds of tissue blanks from 0.5 g rat brain were studied One blank I was obtained by oxidative destruction of the catecholamines at a highly alkaline pH the other blank II by the addition of an oxidant (see table XXI) After five chromatographies 52 cpm (I) and 13 cpm (II) were found in the adrenaline triacetate spot and 13 cpm (I) and 0 cpm (II) in the noradrenaline triacetate spot (above backgrounds). With an overall recovery of 5% this corresponds to 2 ng (I) and 0.4 ng (II) apparent adrenaline and 0.4 ng (I) and 0 ng (II) apparent noradrenaline in the 0.5 g rat brain None of these tissue blanks can be regarded as entirely satisfactory however since both involve a chemical change with a risk that possible contaminants may also be destroyed

Variable amounts of ^3H-impurities even after repeated chromatographies may reduce the accuracy of the method in determinations of small amounts of

Table XXI
Incomplete disappearance of ^{3}H-activity from the adrenaline (A) and noradrenaline (N) triacetate spots in repeated chromatography of blank solutions. In the water blank bidistilled water was "acetylated" In the tissue blanks extracts of 0.5 g rat brain in perchloric acid were processed exactly according to "Methods" and the catecholamines were destroyed after the alumina step In tissue blank I the eluate was allowed to stand for a few days at pH 12 at room temperature. In tissue blank II the catecholamines were oxidized at pH 6.0 with potassium ferricyanide (v Euler and Floding 1955) After acetylation with acetic ^{3}H anhydride (100 Ci/mol) unlabelled carriers were added Chloroform extraction and repeated chromatography was performed as described under "Methods"

Blank	Spot	cpm ^{3}H-activity after chromatography				
		S_1	S_2 S_3	P_4	S_5	P_6
Water	A	32.000	4.000	945	95	121
	NA	13.000	3.800	570	80	32
Tissue I	A	72.000	4.000	232	80	52
	NA	55.000	3 400	420	42	23
Tissue II	A	90.000	3.600	340	75	13
	NA	96.000	6.500	480	55	0

catecholamines. If the remaining ^{3}H-impurity is large it is usually not overlooked since it prevents stabilization of the $^{3}H/^{14}C$ ratio in the sample This leads to further chromatographic purification or if this is not possible the sample is discarded However it cannot be entirely excluded that the $^{3}H/^{14}C$ ratio in a sample can remain constant through 2 3 chromatographies even if the sample still contains a small ^{3}H-impurity This requires that the impurity has an Rf value close to the catecholamine triacetate in several chromatographic systems. This might have been the case for the ^{3}H-impurity found in the adrenaline triacetate spot in the water blank sample after the 4th and 5th chromatography in table XXI According to our experience the small amounts of ^{3}H-impurities that may be present in a sample after 4 6 chromatographies vary considerably even between blanks processed in parallel Therefore it probably does not pay to run a single blank and to subtract this value from the calculated values in the samples Evidently however the amount of a catecholamine determined in a sample by the present technique may be up to 2 4 ng too high because of remaining ^{3}H-impurities.

If higher specific activities of the ^{14}C-catecholamines had been available this would have permitted further purification in critical samples, and allowed higher accuracy by reducing the amount of remaining ^{3}H-impurities Further trials with other purification steps may also solve this problem We have tried to use second derivation (acetylation or silylation) as an early purification step after the radioactive acetylation in order to reach a constant $^{3}H/^{14}C$ ratio

faster but without success. A reduction of the amount of acetic ^3H anhydride used also very probably would reduce the blank values. A preliminary study has shown that this should be possible if the acetylation is performed in a phosphate buffer or if aluminium complexones are used (see p 25).

Part 2

DOUBLE ISOTOPE DILUTION TECHNIQUE WITH COMPLETE ACETYLATION OF CATECHOLAMINES (PREPARATION OF TETRAACETATE)

Chapter VI

TETRAACETYLATION METHOD

Bretschneider (1946) described a study in which adrenaline was acetylated with acetic anhydride in a dry environment in the presence of pyridine whereupon the tetraacetyl derivative was formed i.e. complete acetylation of amino- and hydroxyl groups was obtained. Bretschneider's acetylation technique was taken up by *Brooks and Horning (1964)* and by *Frère and Verley (1967)* in studies of catecholamine derivatives suitable for gas chromatographic separation. Since at the start we considered this technique to be the most promising because of its relatively mild condition we have studied it carefully. We have found that with this method tetraacetylation on a microscale can result in an essentially homogenous product and several systems for chromatographic separation of tetraacetylated catecholamines have been developed. However it has been found difficult to keep the amount of acetic anhydride required as low as desirable in acetylation of tissue extracts with this technique despite trials of various methods including studies in a moisture free environment drying of acetylation solvents etc. For routine use we now therefore recommend the triacetylation technique described earlier. Nonetheless a second method of acetylation may be of interest as a check on results obtained by the triacetylation technique either a second acetylation of the triacetate to tetraacetate (see p. 42) can be performed or parallel analyses can be carried out by the tri- and tetraacetylation methods. *Matthias and Liemann (1970)* have recently described a double-isotope technique similar to ours for determination of catecholamines, employing the tetraacetylation method. Franklin and Mayer (1968, 1969) in a study carried out during the same period as our investigation have also performed acetylation of catecholamines in a

double-isotope technique using acetic anhydride in the presence of pyridine as catalyst. In their study however the acetylation took place with the catecholamines still absorbed to the alumina and according to the authors triacetates of adrenaline and noradrenaline were obtained In our own studies with this technique the acetylation yield was poor and we mostly obtained completely acetylated derivatives of adrenaline and noradrenaline

Methods

The individual steps of the analytical procedure are

A. Preparation of extracts
B Adding of radioactive tracers
C Isolation on alumina.
D Concentration of the eluate
E Acetylation
F Adding of carriers
G Extraction of catecholamine tetraacetates
H I Separation and purification of tetraacetates by chromatography
J Radioactive measurements
K Standards.
L. Calculations.

Materials see Method section I

A-C Homogenization precipitation of proteins from tissue extract and primary isolation over alumina after the addition of ^{14}C tracers are performed in the same way as in the triacetate technique Preparation of the alumina and purity control of tracer catecholamines are described in part 1

D Concentration of the eluate after elution of the alumina with 0 1 N formic acid the eluate is freeze-dried in a 10 ml pear-shaped glass flask with an ungreased ground-glass stopper In certain experiments the final freeze-drying of the last approximately 100 μl of the eluate took place in small glass ampoules (volume about 150 μl). At the end of the freeze-drying the glass flask or the ampoule is filled with nitrogen gas dried over H_2SO_4 and transferred to a plastic glove bag (I^2R Cheltenham Penna)

E Acetylation the acetylation is performed in this glove bag in a dry nitrogen atmosphere The acetylation takes place with 10 20 μl freshly distilled (see part 1) acetic ^3H anhydride (not diluted with benzene) in the presence of pyridine as catalyst (5 volumes freshly distilled pyridine A.R per volume acetic

anhydride) The flask is tightly stoppered or the ampoule sealed after which the process is allowed to continue for 2 hours at 70°C The acetylation is terminated by the addition of 1 ml distilled water The sample is left to stand for 30 min

F Adding of carriers approximately 100 μg unlabelled catecholamine tetraacetate carriers are added (Preparation of carriers see below)

G Extraction the acetylated catecholamines are extracted by vigorous shaking for 10 min with ? ml chloroform The chloroform extract is washed by shaking for 5 min with 2 x 2 ml distilled water after which it is dried in a current of air

H I Separation and purification of the tetraacetates by chromatography Chromatography systems

S_{14} S_{15} Acetic acid water (3 100) (S_{14}) Chloroform (S_{15}) Two-dimensional silica gel TLC

S_8 Chloroform acetic acid (4 1). Silica gel TLC

S_2 P_4 S_5 P_4 as described on page 11

Preparations of the TLC plates pretreatment of the chromatography papers identification of the spots and elution from the chromatograms are performed as described in part 1 (p 12)

Procedure the catecholamine acetates are dissolved in 200 μl methanol and chromatographed on a two-dimensional silica gel plate in system S_{14} S_{15} System S_{14} is used to purify the catecholamine tetraacetates from remaining acetic ^{3}H acid which migrates to the first front Chromatography in *chloroform (S_{15}) in the second direction* reaccumulates the catecholamine acetates at the second front As the second chromatographic step system S_8 is used This system gives a good separation of the different tetraacetates formed They are then eluted separately and further purified in system S_2 P_4 until constant $^3H/^{14}C$ ratios are obtained as described on p 13

J L Radioactive measurements, preparation of standards and calculation were performed according to the technique described in part 1

Preparation of catecholamine tetraacetate carriers

200 mg catecholamine is added to a mixture of 1.5 ml acetic anhydride 3 ml pyridine and 1 ml benzene The acetylation process is allowed to continue for 2 hours at 70°C and is then terminated by the addition of 4 ml water The catecholamine tetraacetates are extracted by shaking for 10 min with 8 ml

chloroform The extract is washed with 3 x 8 ml water and then concentrated in a current of air The wet residue is freeze-dried The catecholamine tetraacetate is dissolved in 0.5 ml methanol and purified by chromatography on a preparative plate in system S_4 Elution is performed with 2 x 5 ml methanol After concentration in a current of air the carrier is kept at −20°C

Rationale of the individual steps

1 Acetylation

As in the triacetylation technique primary isolation of the catecholamines over alumina is necessary to reduce the amount of acetic anhydride required and to minimize the problems of removal of irrelevant tissue products that may be acetylated Here also it was found of importance to elute the catecholamines from the alumina with an acid sufficiently weak to avoid too great interference with the acetylation. As alternative primary purification steps ion-exchangers (Dowex or Amberlite columns) were tried but these were found unsuitable owing to the large amounts of salts from the columns that accumulated in the eluate

At the tetraacetylation a very poor recovery was obtained if the eluate was not dried completely and a homogenous acetylation product could not then be attained In an attempt to achieve a maximal recovery in the acetylation technique with as little destruction of the acetic anhydride as possible a method was developed in which the acetylation took place in a moisture free environment in a plastic glove bag with nitrogen gas which had been dried over concentrated sulphuric acid All pipetting and stoppering of the acetylation flasks was done in these glove bags.

An attempt to find the optimal ratio of acetic anhydride to pyridine the optimal reaction temperature and reaction time (see tables XXII XXIV) showed that the amount of pyridine was not especially critical but that an acetic anhydride/pyridine ratio of 1:4 (vol/vol) gave the best acetylation recovery The acetylation process could be hastened considerably and the recovery improved by raising the temperature A temperature of 70°C and an acetylation time of 2 hours gave optimal recovery No greater destruction of catecholamines was observed at the raised acetylation temperature than in acetylation at room temperature In order to prevent destruction of catecholamines by possible oxidation at the acetylation however we tested the addition of a reducing agent N-acetyl-cysteine at the acetylation but this gave no appreciable increase in the recovery

Despite the experimental precautions described above a large excess of acetic anhydride was required for the conversion of at least 50% to tetraacetate If pure catecholamines (without the presence of residues from tissues or alumina) were tetraacetylated a minimal concentration of about 0 1 M acetic anhydride in the reaction mixture was necessary (see fig 10) This is 10 times more than in the triacetylation technique The total volume of the reaction mixture determines the amount of acetic anhydride required With our technique the minimal total volume was found to be 50 μl Smaller volumes gave lower recoveries probably due to losses of catecholamines on the walls of the reaction vessel.

Table XXII
Recovery of pure ^{3}H-isoprenaline (5 10^{-4} μmol) as tetraacetate after acetylation with 0.2 M acetic anhydride in the presence of varying amounts of pyridine at 37°C for 24 h. Dry benzene was added to all samples to give a total reaction volume of 50 μl. The percentage values for recovery of ^{3}H activity in the tetraacetate spot after chromatography in S_8 are given.

	Acetic anhydride pyridine (vol/vol)				
	1:1	1 4	1 9	1 50	1 150
% tetraacetate recovery	6	85	67	73	47

Table XXIII
Recovery of pure ^{3}H-adrenaline (1 10^{-5} μmol) as tetraacetate after acetylation with 0.06 M acetic anhydride for 24 h at varying temperatures. Acetic anhydride pyridine 1:4 (vol/vol) Dry benzene was added to give a total volume of 50 μl. Th percentage values for recovery of ^{3}H activity in the tetraacetate spot after chromatography in S_8 are given.

	Acetylation temperature		
	37 C	50°C	70 C
% tetraacetate recovery	22	29	43

Table XXIV
Recovery of pure ^{3}H-adrenaline (3 10^{-5} μmol) as tetraacetate after acetylation with 0 4 M acetic anhydrid at 70 C for different times. Acetic anhydrid pyridine 1:4 (vol/vol) Dry benzene was added to give total volume of 50 μl The percentage values for recovery of ^{3}H activity in the tetraacetate spot after chromatography in S_8 are given.

	Acetylation time		
	1/2 h	2 h	24 h
% tetraacetate recovery	7	83	83

Acetylation vessels of different materials were tested With the same composition of the reaction mixture acetylation in siliconized glass flasks and in Teflon® polethylene or vinyl ampoules gave a poor recovery as compared to Pyrex® glass flasks The best result was obtained with freshly prepared glass ampoules (volume about 150 µl) of ordinary soda glass which were kept sealed during the acetylation process. Compared with acetylation in our usual conical glass flasks (Pyrex®) volume 5 10 ml a somewhat smaller amount of acetic anhydride was required in the ampoules and the acetylation recoveries were more constant

On acetylation under the above conditions, predominantly tetraacetate is formed though with a varying admixture of triacetate As seen in table XXV very large concentrations of acetic anhydride are required to attain nearly complete tetraacetylation Tetra- and triacetates of the catecholamines studied in this investigation noradrenaline adrenaline and isoprenaline as well as the triacetate of dopamine can be separated chromatographically however (see table VI) and incomplete acetylation although constituting a problem is therefore no direct impediment to the determinations if several different chromatographic systems are combined To illustrate this point, table XXVI shows the tetraacetylation of ^{14}C-adrenaline with two different concentrations of unlabelled acetic anhydride extraction into chloroform drying and chromatography of the chloroform phase Using 0 13 M acetic anhydride a considerable triacetate fraction was seen When 1.66 M acetic anhydride was used almost pure tetraacetate was obtained In fraction (2 3) there was a small peak less than 1% of the tetraacetate activity in both samples It probably consisted of di and monoacetate of the catecholamine No other distinct peaks could be distinguished which would have indicated that the catecholamines were destroyed in the acetylation procedure and that breakdown products were acetylated

Table XXV
Recovery f p re ^{14}C-adrenaline (5.5 10^{3} µmol) a tetraacetate and triacetate after acetylation with varying concentration of acetic anhydride at 70°C for 2 h Acetic anhydride pyridin 1 4 (vol/vol) Dry benzene was added to all samples to give a total volume of 50 µl The percentage values for recovery of ^{3}H activity in the tetra- and triacetat spot after chromatography in S_8 are given

% recovery	Molar conc acetic anhydride				
	0 06	0 12	0 18	0.24	2.0
tetraacetat	49	81	88	87	85
triacetat	23	11	5	5	<1

Table XXVI
Distribution of ^{14}C-activity in silica gel chromatograms (from starting line to front) following tetraacetylation of 1 μg ^{14}C-adrenaline with 0.13 M (sample 1) and 1.66 M (sample) acetic anhydride. Chromatography of the chloroform extract in S_4. The ^{14}C-activities are given in per cent of the total activity of the chromatogram.

Sample	Fractions in cm from starting line										
	0-1	1-2	2-3	3-4	4-5	5-6	6-7 A-triac	7-8	8-9 A-tetraac	9-10	10-12
1 % ^{14}C activity	0.21	0.18	0.30	0.18	0.16	0.28	11.13	4.11	81.76	1.01	0.48
2 % ^{14}C activity	0.37	0.27	0.61	0.20	0.37	0.57	1.11	0.91	95.09	0.31	0.18

2. Termination of acetylation and extraction

Since a very large excess of acetic ^{3}H anhydride must be used in acetylation by the tetraacetate technique it was necessary to find a way of separating the catecholamine tetraacetates formed from other ^{3}H-labelled products as quickly as possible. The excess acetic anhydride can be converted to acetic acid by a reaction with water or to ethyl acetate with ethanol. Since the tetraacetates of catecholamines are hydrophobic they can be extracted in a solvent with a low polarity and any ethyl acetate formed can be volatilized. Different extraction systems were studied with respect to loss of tetraacetate, removal of unacetylated catecholamines and residuals of the excess of acetic anhydride.

To an acetylation mixture (2 μl non-labelled acetic anhydride, 8 μl pyridine and 40 μl benzene containing no catecholamines) 1 ml water was added. This is the usual way to terminate an acetylation. Then tracer amounts of ^{14}C-isoprenaline tetraacetate in methanol and ^{3}H-adrenaline in acetic acid were added. By shaking with 2 ml chloroform the tetraacetate could be extracted to almost 100% while less than 1% of unacetylated adrenaline was extracted into the organic phase. When dichloromethane instead of chloroform was used a little more unacetylated adrenaline was extracted.

In another experiment 1 ml of water was added to each of a number of tubes containing acetylation mixtures (2 μl acetic ^{3}H anhydride, 8 μl pyridine, 40 μl benzene). Thirty minutes after addition of the water, when all acetic anhydride should have been hydrolyzed, extraction was started with 2 ml of one of the following solvents: chloroform, dichloromethane, carbon tetrachloride and isooctane. In all cases some radioactivity passed into the organic phase. Chloroform and dichloromethane took up the least. Using 25% ethanol instead of water to terminate the mock acetylation made no difference. After washing of the chloroform with 2 x 2 ml water the uncomfortable amount of 0.4%

(about 1.2 million cpm!) of the ^{3}H-activity originally present in the reaction mixture was still in the chloroform Instead of extensive further washing of the organic phase we found that by chromatography on silica gel in acetic acid water (3 100) (S_{14}) the acetic ^{3}H acid formed and other polar ^{3}H-labelled impurities could be separated effectively from the tetraacetate spots. Of the ^{3}H-activity in the chloroform phase which did not arise from the tetraacetate formed 99.8% was found at the leading edge of the chromatogram, while the catecholamine tetraacetates migrated only very slightly but with a strong tendency to tailing. By further chromatography in a second direction in pure chloroform (S_{18}) however the catecholamine tetraacetates could be reaccumulated and eluted for further separation in other chromatographies By means of this technique tetraacetylated catecholamines can be rapidly freed from ^{3}H-residuals and in the subsequent chromatographies constant ratios can be obtained relatively quickly

3 Chromatographic isolation

As mentioned above on tetraacetylation of catecholamines varying amounts of triacetate are also formed unless a very large excess of acetic anhydride is used A combination of several different chromatographic systems is required to ensure removal of the triacetates. Several different systems have been developed (see table VI) Elution from the chromatograms can be done with methanol or chloroform as in the triacetate technique The loss of tetraacetate during purification to constant ratios is 20 30% per chromatography

4 Stability of catecholamine tetraacetates

The stability of the catecholamine tetraacetates is comparable with that of the triacetates They are essentially stable in organic solvents, while in aqueous solutions they are rapidly hydrolysed at basic and highly acid pH Solutions of adrenaline noradrenaline and isoprenaline tetraacetate in methanol have been stored in a refrigerator for up to 1 month without appreciable destruction

5 Tetraacetylation in biological materials

In an experiment shown in fig. 10 we compared the tetraacetate recoveries after acetylation in biological extracts pure alumina "eluates" and 0 1 N formic acid Perchloric acid extracts of rat brain (0.5 g) rat heart (0.5 g) human plasma (10 ml) and human urine (20 ml) were passed over alumina elution was performed with 0 1 N formic acid 1 μg ^{14}C-adrenaline was added and the

eluates were freeze-dried. In the pure alumina "eluates" a mock extract without tissue was passed over alumina and then treated as the true extracts. In the no-alumina case [14]C adrenaline was added to formic acid and was only freeze-dried. Acetylation took place with varying concentrations of acetic anhydride in the presence of pyridine as catalyst (acetic anhydride : pyridine 1:4). Benzene (dried over metallic sodium) was added in some of the samples to give a final volume of at least 50 μl in all samples. The adrenaline tetraacetate formed was extracted in chloroform and chromatographed in system S_8. The per cent recovered [14]C activity in the tetraacetate spot is given in the figure.

It is evident that considerably more acetic anhydride was necessary for the tetraacetylation of adrenaline in biological extracts after primary isolation on alumina than for acetylation of pure catecholamines. Tetraacetylation in the brain and heart extracts however required about the same concentrations of acetic anhydride as in pure alumina "eluates". The recoveries were somewhat lower in extracts of plasma and urine. Obviously, as in the triacetylation technique (see p. 23), it is residues from alumina (aluminium salts or aluminium hydroxide) that are the most important disturbing factors in the tetraacetylation. In the plasma and urine samples however some other

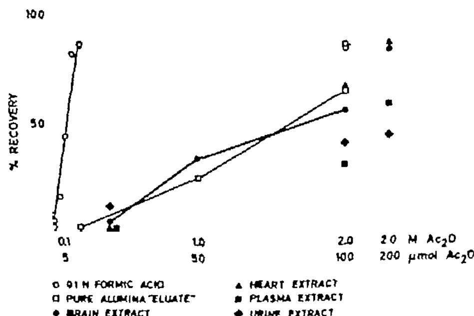


Fig. 10. Recovery from biological extracts, pure alumina eluates and pure formic acid solutions of [14]C-adrenaline (1 μg) as tetraacetate after tetraacetylation with varying amounts of acetic anhydride. The per cent recovered [14]C-activity after chloroform extraction and chromatography in S_8 is given.

unknown inhibiting substances might also have been present These have not yet been investigated further The inhibition caused by alumina can be partly overcome by increasing the total amount of acetic anhydride pyridine mixture without changing the molarity of the acetic anhydride as is shown in fig 10 The isolated points to the right in the figure are recoveries obtained with 50 μl acetic anhydride the points at the end of the lines were obtained with 10 μl of the same solution The concentration of acetic anhydride in the reaction mixture in both cases was 2 M The explanation is not evident It is not probable that the inhibition is due to impurities which consume acetic anhydride Extrapolation of the lines to 0 recovery shows that at most 10 μmol are lost It is hard to believe that this amount which corresponds to a 10% reduction in concentration could reduce the recoveries to the extent seen in a comparison between the two groups of points at the right hand It is therefore more probable that the dilution of the impurities caused by a large volume of reaction mixture is of importance

It is obvious that at least 100 μmol (about 10 μl) acetic anhydride is required for attainment of acceptable tetraacetylation of catecholamines in extracts from biological materials This is 5 10 times more than in the triacetylation technique Even when these high amounts of acetic anhydride are used one often gets an incomplete acetylation a mixture of tetra and triacetates which increases the problems of chromatographic isolation

In an attempt to reduce the acetic anhydride requirement for tetraacetylation of catecholamines in biological materials we tried other techniques for the primary isolation of the catecholamines prior to acetylation For this ion-exchange chromatography on a Dowex or Amberlite column as well as extraction in butanol as described by Shore and Olin (1958) were studied However all these methods gave less good recoveries than if alumina was used as the primary isolation step

We also tried to improve the tetraacetylations of catecholamines that had been isolated on alumina by increasing the acetylation time but this did not give any better results nor did an attempt to increase the amount of pyridine in the reaction mixture However preliminary experiments with aluminium complexones seem more promising.

Double isotope experiments with the tetraacetate technique

Table XXVII presents the results of some experiments in which standard solutions of adrenaline and isoprenaline in aqueous solution were measured after the addition of ^{3}H-labelled tracer catecholamines by acetylation with

acetic ^{14}C anhydride (specific activity 1 10 Ci/mol) according to Bretschneider's technique The total amount of catecholamine in the samples was calculated from the constant ratios

Since we have found that acetylation by the triacetylation technique described by Welsh offers advantages in many respects for double isotope determinations, we have performed no further experiments on unacetylated catecholamines by the tetraacetylation technique The latter method has been used extensively however for second acetylation of catecholamine triacetates as described previously

Table XXVII

Determination of known amounts of isoprenaline (Iso) and adrenaline (A) by the tetraacetate double isotope technique Italicized ratios were utilized in the calculations CA catecholamine

Unlabelled CA subst	μg	Tracer ^3H-CA μg	$^3H/^{14}C$ ratio after chromatography S_1	$S_{1+}S_4$	S_2	P_4	S_5	S_7	Mean ratio	Unlabel. CA recov μg
Iso	1.0	0.01	7.2	9 7	*10.3*	*10.[illegible]*	*10 0*		10.17	1 01
Iso	0.2	0.01	7.3	8.8	*9.[illegible]*	*9.[illegible]*	*9.3*		9.23	0.21
A	1 0	0 02	4 4	10 8	10.4	*11 7*	*11.3*	*11.[illegible]*	11 40	0.93
A	1.0	0 02	–	9.3	10.2	*11 7*	*11 0*	*11 0*	11.23	0.96
A	0.1	0.02	2 4	8 7	*9.3*	*9.3*	*9.2*	*9 6*	9.35	0.099

Part 3

DISCUSSION AND CONCLUSIONS

Chapter VII

THE ACETYLATION METHODS

In the hands of the masters (Vendsalu 1960 Häggendal 1963 Saito Woronkow and Myers 1969 O'Hanlon Jr., Campuzano and Horvath 1970 Renzini Brunori and Valori 1970 Griffiths, Leung and McDonald 1970) very good sensitivity in the determination of catecholamines has been attained with the THI fluorescence method However the quantitative differentiation of adrenaline and noradrenaline when one of the compounds is present in a much larger amount than the other still remains a problem A number of different modifications of the THI method have been developed to overcome this problem (see review by Häggendal 1966) but none is ideal if the concentration of one of the catecholamines is very low (Häggendal 1966 Iversen 1967) Our long and indeed laborious work to develop a double isotope dilution method for catecholamine determination was due to an interest in the biological importance of "minority" catecholamines.

Among different possible catecholamine derivatives for double isotope studies we found that acetyl derivatives should be most suitable since radioactive acetic anhydride could be obtained with high specific activity and earlier studies by Bretschneider (1946) and Welsh (1952 1955) had shown that acetylation of catecholamines could proceed to a fairly homogenous product In further studies of the literature it was found that in a brief report as early as in 1959 Goldstein Friedhoff and Simmonds had pointed out the possibility of assaying catecholamines quantitatively by the double isotope technique by acetylation with acetic anhydride The authors seem not to have pursued the practical application of this technique further however

Acetylation of catecholamines has been employed in order to obtain more stable products that can be separated chromatographically for primary isolation of catecholamines prior to more unselective quantitative methods of determination The procedure was used by Welsh (1955) for colorimetric

determination of noradrenaline and has since been taken up by several groups of workers to increase the selectivity of fluorometric determinations of catecholamines by the unselective ethylenediamine condensation method (Goldstein Friedhoff and Simmonds 1959 Laverty and Sharman 1965 Guldberg and Yates 1968 1969 Crawford and Yates 1970 Callingham and Sharman 1970) Goldstein *et al.* (1960 1961 1962) as well as a number of other groups (Hagopian Dorfman and Gut 1961 Hempel and Männl 1969) have used the acetylation procedure in several studies for chromatographic separation of different metabolites from radioactive precursors All these authors have employed the triacetylation technique as described by Welsh (1952 1955) Bretschneider s tetraacetylation technique (1946) has been used more seldom but as mentioned above it was employed in gas chromatographic studies by Brooks and Horning (1964) and Frère and Verley (1967)

In the present study the catecholamine tri and tetraacetates were found to be stable enough to permit extensive purification This gives a possibility to determine each catecholamine separately with the double isotope technique Although great and varying losses may occur in connection with the acetylation and the chromatographies this can be compensated for by an early addition of radioactive tracers. The possibility of adding large amounts of carrier catecholamine acetates prior to the chromatographic separations considerably decreases the losses. Since an absolute recovery is not essential in the double isotope technique more effective purification can be achieved than in a fluorometric method Further the isotope ratio gives a possibility to follow the purification of each catecholamine individually A constant isotope ratio increases the certainty that a pure substance is in fact being assayed without interference from other substances with similar Rf values in the chromatography The possibility of ensuring further (by second derivation or crystallization) that a pure substance has actually been isolated prior to the quantitative determination is also a considerable advantage Thus in comparison with the conventional fluorescence techniques a more effective separation of different catecholamines in a sample may be attained with our technique This *makes it possible to determine small* amounts of *e.g.* adrenaline even *in the* presence of at least 100 times more noradrenaline It should also be possible to determine other catecholamines simultaneously present in very different concentrations. Of special interest are derivatives which cannot form trihydroxyindole and/or be made to fluoresce by the Falck-Hillarp technique N-methyladrenaline (Axelrod 1960) is a case in point Also several of the catecholamine metabolites should be suitable for the method In this case however suitable primary isolation steps, before acetylation will have to be further developed

The sensitivity of our method conservatively rated at present lies around 1 10 ng depending on whether ^{14}C or ^{3}H-acetic anhydride is used Compared to the fluorescence methods in the hands of experts it does not offer any appreciable increase in sensitivity when applied to determinations of single catecholamines. It may however be of use in critical experiments owing to its high degree of selectivity This may be of particular value when other substances with fluorescence of their own are present in the tissue Carruthers *et al.* (1970) have recently shown that several common dietary and therapeutic agents may cause strikingly disturbing fluorescence in the THI fluorescence technique

The sensitivity of the method depends on the specific activity of the ^{14}C-catecholamine tracers or the acetic ^{14}C anhydride It should be possible to increase the sensitivity once really high specific activities become available Another possible technique that we have discussed briefly above is to perform acetylation with bromoacetic anhydride followed by neutron activation of the sample after the final chromatographic separation If ^{3}H-catecholamine tracers were used this might give a very high sensitivity

The greatest disadvantage of the method is that since it requires such a long chromatographic purification process it is time-consuming Relatively few samples can be analysed simultaneously at least if several catecholamines are to be determined Another problem is the high cost of the determinations especially if acetic ^{14}C anhydride with high specific activity is used

In certain cases, foreign substances can be expected to cause interference in the acetylation technique as is evident from the finding of N-acetylnor metanephrine N-acetyldopamine and N-acetylmethoxydopamine in the urine of rats treated with monoamine oxidase inhibitors (Smith and Wortis 1960 Goldstein and Musacchio 1962) Similary N-acetyldopamine N-acetylnor methanephrine and N-acetylmetanephrine has been found in the urine of patients with neuroblastoma or pheochromocytoma (Sekeris and Herrlich 1963 Hanson and v Studnitz 1965 Borud and Gjessing 1970) O'Gorman *et al.* 1970 found small amounts of N-acetyldopamine and N-acetyl 3-methoxy-4-hydroxyphenylethylamine in the urine from patients with Parkinson's disease receiving high amounts of L-DOPA Tyce (1971) in studies of the metabolism of DOPA in isolated perfused rat liver found a rather high amount of N-acetyldopamine in the bile Under normal conditions, however Borud and Gjessing (1970) found no measurable amounts of N-acetylated catecholamines in human urine

Obviously the presence of N-acetylated catecholamines can give rise to false results in the acetylation technique After tri- and tetraacetylation these pre-acetylated amines will be included with the pre-unacetylated ones. For

instance N acetylnoradrenaline thereby contributes 2/3 or 3/4 of its true amount to the amount of noradrenaline measured

The problem of isotope fractionation, as already discussed is important to keep in mind but constitutes no obstacle to the use of the method as long as it is not overlooked

The specific activity of the tracers and radioactive reagents used has to be known in a double isotope technique Since the catecholamines are unstable and since high specific activity compounds are subject to radiolysis very frequent determinations of specific activity are necessary The procedure with two standards used in the present method is in fact equivalent to a redetermination of the specific activities in each analytical run

Chapter VIII

ALTERNATIVE APPROACHES

The need for an improved method for determination of catecholamines has led to parallel development work in this field in many different laboratories and during recent years several reports on double isotope techniques have been presented. The use of acetylation techniques similar to ours by Matthias and Liemann (1970) and Franklin and Mayer (1968, 1969) has already been mentioned (p. 73).

Aizawa and Yamada (1969) have suggested a technique in which a double isotope labelled noradrenaline derivative is formed by a reaction with p-toluenesulphonyl chloride ^{35}S. This technique cannot be evaluated as yet owing to the lack of details and results in their very brief report. Another very interesting technique has been presented recently by Blaedel and Anderson (1971) who oxidized noradrenaline and adrenaline with radioactive iodine (^{125}I) to iodo-adrenochrome. The authors used this method for determination of adrenaline and noradrenaline in buffer solutions and in some experiments for determination of noradrenaline in urine. They used a reverse isotope dilution technique but the method should also be applicable in the form of a double isotope technique. It should have the advantage that ^{3}H-labelled catecholamines, with their very high specific activity, could be used as tracers. Although this technique looks very promising according to the results published up to now the sensitivity is still poor owing to the very low recovery on oxidation and separation, e.g. for iodo-adrenochrome only 0.5%. The separation of the derivatives formed, e.g. from adrenaline and dopamine, is also poor as yet.

Saelens, Schoen and Kovacsics (1967) presented the first practicable radiochemical technique for catecholamine assay. This is based on enzymatic transfer of ^{14}C-methyl groups from S-adenosyl-methionine-methyl-^{14}C by phenyl-ethanolamine-N-methyl-transferase (PNMT) to noradrenaline. The technique was originally developed for assay of noradrenaline alone, but Molinoff and Axelrod (1969) have shown that it should be useful for several β-hydroxylated phenylethanolamines, e.g. adrenaline and octopamine. Iversen and Jarrot (1969) found that this method gave noradrenaline values in several tissues that agreed fairly well with those of the fluorometric methods. In certain tissues, e.g. the brain, however, the reaction does not seem to be complete and the values obtained are therefore uncertain. The authors discuss

the possibility of overcoming this problem by using the technique as a double isotope method with the addition of radioactively labelled tracers

An alternative enzymatic method was presented by Engelman Portnoy and Lovenberg (1968) who converted the catecholamines to their ^{14}C-O methylated derivatives with S-adenosyl-methionine-methyl ^{14}C in the presence of catechol-O-methyl-transferase Engelman and Portnoy (1970) have demonstrated the application of the technique to plasma samples using a double-isotope procedure Since this enzyme is less substrate-specific than PNMT (Axelrod and Thomchick 1958) it should be possible to develop the method further for determination of a large number of catechols Sato and DeQuattro (1969) for instance have shown its use for assay of dihydroxymandelic acid Engelman and Portnoy (1970) have also found that their technique allows the assay of adrenaline and noradrenaline in the same sample even when there are large differences in the concentrations Both the enzymatic techniques require careful isolation of the catecholamine derivatives formed to ensure reliable results which means that like our method they are rather time-consuming.

Several attempts to develop suitable gas chromatographic techniques in order to improve the catecholamine determinations have also been made during the 1960's Gas chromatographic separation of non-derivated catecholamines has been reported by several groups (Fales and Pisano 1962 Parker Fontan and Kirk 1962). More effective separation has been obtained however with different more stable catecholamine derivatives. The derivation methods most often used are formation of a Schiff base (Brochmann-Hanssen and Baerheim-Svendsen 1962) formation of trimethyl silyl ethers (Sen and McGeer 1963 Lindstedt 1964 Kawai *et al.* 1966 Horning Moss and Horning 1967) acetylation (Brooks and Horning 1964 Frère and Verley 1967) silylation of hydroxyl groups in combination with the formation of Schiff base or oxazolidine including the amino group (Capella and Horning 1966) or with acetylation of the amino group (Horning *et al.* 1968) acetylation of hydroxyl groups and silylation of the amino group (Brochmann-Hanssen and Baerheim Svendsen 1962) and formation of cyclic boronate (Antony Brooks and Middleditch 1970) The problem of instability of the catecholamines and several of their derivatives at raised temperature has meant that it has not yet been possible to use the gas chromatographic methods with any degree of certainty and that they have not given the desired sensitivity in quantitative assays of catecholamines in biological material The combined gas chromatography-mass spectrometry technique has been studied by for instance Antony *et al.* (1970) and Änggård and Sedvall (1969) Lately Narasimhachari and Vouros (1972) described the possibility to use isothiocyanate derivatives

for gas chromatography and mass fragmentography. The possibility of considerably increasing the sensitivity in gas chromatographic separations by combination with the electron-capture detector technique has been studied in several laboratories during the last few years. Several halogenated reagents have been found suitable for catecholamine derivation, e.g. trifluoroacetic anhydride (Clarke *et al.* 1967, Kawai and Tamura 1968, Änggård and Sedvall 1969), pentafluoropropionic or heptafluorobutyric anhydride (Änggård and Sedvall 1969), N-heptafluorobutyryl imidazole (Horning *et al.* 1968, Cummins and Fourier 1969), pentafluorobenzaldehyde (Moffat and Horning 1970) and 2,4-dinitrofluorobenzene (Walle 1968). A few studies employing this method on biological material have been carried out, e.g. by Clarke *et al.* (1967) who assayed dopamine in urine. The very high sensitivity that should be obtained theoretically with this method has not been reached hitherto, however, owing to several problems with the electron-capture analysis technique, such as derivative and detector instability and problems of interference from other substances in biological material.

Some interesting reports on attempts to increase the sensitivity of fluorometric methods by other procedures than the conventional have recently been presented. Ritzén (1966) and van Orden (1970) have shown the possibility of quantitative histochemical studies of biogenic amines by microspectrofluorometry of formaldehyde-induced fluorescence. This technique encounters the difficulty that extracellulary bound catecholamines probably give higher fluorescence than granule-bound ones. Jonsson and Malmfors (1966) have described a highly original method with the possibility of assaying noradrenaline in as small amounts as 1 pg. It is a combination of a biological *in vitro* technique and microfluorometric determination of formaldehyde fluorescence. Noradrenaline from the extract is taken up by the adrenergic nerve endings of rat iris and the amount of noradrenaline is then determined microfluorometrically. Since the membrane pump in sympathetic neurones is not completely specific, however, dopamine and adrenaline as well as other substances can interfere with the determinations.

Chapter IX

CONCLUSIONS AND SUMMARY

Several catecholamines and their metabolites can be acetylated on a microscale with good yield Adrenaline and noradrenaline can be converted either to their triacetyl derivatives with a free OH group on the β-carbon atom or to their tetraacetyl derivatives with complete acetylation of amino- and hydroxyl groups. The consumption of acetic anhydride is some 10 times less in the former technique which therefore is preferred by us The acetylation methods give a possibility for the quantitative assay of adrenaline and noradrenaline by a double isotope dilution technique Since the acetylation process is a relatively unselective reaction it should be possible to assay even other catecholamines and their metabolites with this technique However this makes a further study of the problem of primary isolation steps before the acetylation necessary

A constant $^3H/^{14}C$ ratio in successive chromatographies on different systems after acetylation of ^{14}C-catecholamine with acetic ^3H anhydride strongly indicates that pure catecholamine triacetate has been isolated. However using the triacetylation technique one gets a further possibility to check the purity of the substance since the free OH group can be used for second derivation (acetylation or silylation) Another possibility of ensuring that the catecholamine derivatives have in fact been isolated from impurities – which is necessary to get reliable determinations – is by crystallization However crystallization of catecholamine acetates take place very slowly which makes this technique difficult

Of great importance in quantitative determinations is that the problem of isotope fractionation is not overlooked The possibility that N-acetyl derivatives of some catecholamines may be formed *in vivo* under certain circumstances must be kept in mind

The greatest advantage of this double isotope technique is the possibility of measuring several catecholamines in the same sample even when there are very large differences in concentration between them The sensitivity is also rather good From a practical point of view we have found the lower limit of sensitivity to be about 10 ng However our technique cannot replace the conventional fluorescence methods for the dominant catecholamines since the double isotope technique is far too time-consuming

It should be possible to further improve the radiochemical techniques for catecholamine assays for instance by the aid of radioactively labelled substances

with higher specific activity It may perhaps even be possible to develop a radioimmunoassay for catecholamine analysis However whether the radiochemical methods will be the techniques of choice in the future is difficult to predict.

Summary

Two double isotope derivative techniques for the quantitative measurement of adrenaline and noradrenaline were developed

Method I determination by the use of 3 4,N-triacetyl derivatives

The main steps of the method were

1 Preparation of extracts homogenization and protein precipitation was performed with 0 4 N perchloric acid No destruction of the catecholamines in this procedure was observed

2 Adding of tracers exactly known volumes from stock solutions of ^{14}C (or ^{3}H) adrenaline and noradrenaline were added The chemical amounts should not be much higher then the expected amounts of catecholamines in the sample

3 Primary isolation of catecholamines absorption of the catecholamines on alumina at pH 8.3 gave the best result The brand of alumina, its pretreatment and handling, the acid used as eluent and the contact time between alumina and eluent were all factors of importance for the recovery at the subsequent acetylation. 0 1 N formic acid was the best eluent

4 Concentrating the eluate freeze-drying of the eluate gave no appreciable losses.

5 Acetylation triacetylation was performed in a saturated solution of $NaHCO_3$ with ^{3}H (or ^{14}C) acetic anhydride Acetylation could also be performed in a buffer solution pH 8.0 8.3 The amount of acetic anhydride should be as low as possible to reduce the costs of the technique and problems of interfering ^{3}H (or ^{14}C) activity

6 Adding of carriers about 100 μg unlabelled adrenaline and noradrenaline triacetates were added as carriers

7 Extraction the triacetyl derivatives were extracted into chloroform

8 Separation and purification the catecholamine triacetates were chromatographed in several solvent systems until constant $^{3}H/^{14}C$ ratios were obtained indicating that pure substances had been isolated A shifting $^{3}H/^{14}C$ intraspot ratio was observed in most chromatograms This was shown to be due to isotope fractionation of an otherwise essentially homogenous substance The spot should be carefully identified before elution to avoid errors due to isotope

Chapter IX

CONCLUSIONS AND SUMMARY

Several catecholamines and their metabolites can be acetylated on a microscale with good yield. Adrenaline and noradrenaline can be converted either to their triacetyl derivatives with a free OH group on the β-carbon atom or to their tetraacetyl derivatives with complete acetylation of amino- and hydroxyl groups. The consumption of acetic anhydride is some 10 times less in the former technique which therefore is preferred by us The acetylation methods give a possibility for the quantitative assay of adrenaline and noradrenaline by a double isotope dilution technique Since the acetylation process is a relatively unselective reaction it should be possible to assay even other catecholamines and their metabolites with this technique However this makes a further study of the problem of primary isolation steps before the acetylation necessary

A constant $^3H/^{14}C$ ratio in successive chromatographies on different systems after acetylation of ^{14}C-catecholamine with acetic-^3H anhydride strongly indicates that pure catecholamine triacetate has been isolated However using the triacetylation technique one gets a further possibility to check the purity of the substance since the free OH group can be used for second derivation (acetylation or silylation) Another possibility of ensuring that the catecholamine derivatives have in fact been isolated from impurities – which is necessary to get reliable determinations – is by crystallization However crystallization of catecholamine acetates take place very slowly which makes this technique difficult

Of great importance in quantitative determinations is that the problem of isotope fractionation is not overlooked The possibility that N-acetyl derivatives of some catecholamines may be formed *in vivo* under certain circumstances must be kept in mind

The greatest advantage of this double isotope technique is the possibility of measuring several catecholamines in the same sample even when there are very large differences in concentration between them The sensitivity is also rather good From a practical point of view we have found the lower limit of sensitivity to be about 10 ng However our technique cannot replace the conventional fluorescence methods for the dominant catecholamines since the double isotope technique is far too time-consuming.

It should be possible to further improve the radiochemical techniques for catecholamine assays for instance by the aid of radioactively labelled substances

ACKNOWLEDGEMENTS

This study has been carried out in the Department of Medical Pharmacology University of Uppsala.

I wish to express my sincere thanks to

My teachers Professor Ernst Bárány and Docent Lars Terenius who throughout the study always have been ready to give expert advice generous support and encouragement.

Docent Dag Lundberg together with whom this work was started and the first years of violent struggle with the method were fought

Mrs. Laine Bennich-Björkman for her skilful technical assistance solidarity and friendship

Mrs. Ann-Marie Kjellberg and Miss Marjut Hostman for skilful secretarial help and Mr Torsten Ekwall for drawing the figures

Mrs. Maud Marsden for her kind help with the translation of the main part of this work into English

The "Sauna group" and the rest of the staff for creating a friendly and cheerful working atmosphere

Dr Peter D Klein Division of Biological and Medical Research Argonne National Laboratory Argonne Illinois, for his kind assistance with the isotope fractionation calculations.

Dr Dag Brune AB Atomenergi Studsvik, for help with the neutron activation.

Mr Rolf Andersson, Department of Chemistry College of Agriculture Uppsala for help with the mass spectrometry

My deep thanks also go to my wife Anna Kajsa and finally I want to thank my son Matts for not having torn all pages of the manuscript to pieces.

The work was supported by grants from the Medical Faculty University of Uppsala and by grants EY–00231 from the National Eye Institute US PHS to Prof Bárány and grant 14X–733 from the Swedish Medical Research Council

REFERENCES

Alzawa, Y and K. Yamada, Double isotope derivative method for determination of noradrenaline in rat brain. *Jap J Pharmacol.* 1969 *19* 474–476

Anggård E and G. Sedvall Gas chromatography of catecholamine metabolites using electron capture detection and mass spectrometry *Analyt. Chem* 1969 *41* 1250–1256

Ansell, G B and M.F Beeson, A rapid and sensitive procedure for combined assay of noradrenaline, dopamine and serotonin in a single rat brain. *Analyt. Biochem.* 1968 *23* 196–206

Anton, A.H and D.F Sayre A study of the factors affecting the aluminium oxide trihydroxyindole procedure for the analysis of catecholamines. *J Pharmacol. exp Ther* 1962 *138* 360–375

Anton A.H and D.F Sayre The distribution of dopamine and DOPA in various animals and a method fo their demonstration in diverse biological material. *J Pharmacol. exp Ther* 1964 *145* 326–336

Antony G.M C.J W Brooks and B.S Middleditch The use of boronate derivatives in the characterization of catecholamines and related β-hydroxyamines by gas liquid chromatography-mass spectrometry *J Pharm. Pharmacol.* 1970 *22* 205–213

Axelrod, J and R. Thomchick Enzymatic O-methylation of epinephrine and other catechols. *J biol. Chem.* 1958 *233* 702–705

Axelrod J., N-methyladrenaline, a new catecholamine in the adrenal gland *Biochim. biophys. Acta (Amst)* 1960 *45* 614–615

Beckett A.H. and N.H Choulis, Multiple spot phenomena using thin layer chrom tography of pure organic bases. *J Pharm. Pharmacol.* 1963 *15* 236T–245T

Bertler Å., A Carlsson and E Rosengren, A method for the fluorometric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand* 1958 *44* 273–292

Bertler Å. and E Rosengren, Occurrence and distribution of catecholamines in brain. *Acta physiol. scand.* 1959 *47* 350–361

Blaedel, W.J and T.J Anderson Radioisotope derivative procedure for determination f epinephrine or norepinephrine. *Analyt Chem.* 1971 *43* 521–529

Boon, P.F.G and A.W Mace The gas chromatographic determination of adrenaline in pharmaceutical products. *J Pharm. Pharmacol.* 1969 *21* Suppl. 49S–51S

Borud O and L.R Gjessing, N-acetylnormetanephrine and N-acetylmetanephrine in urine from patients with neuroblastoma. *Clin. chim. Acta* 1970 *27* 552–554

Bretschneider H Ueber die Acetylierung von Adrenalin. *Mh. Chem.* 1946 *66* 355–367

Brochmann-Hanssen, E and A Baerheim-Svendsen, Separation and identification of sympatomimetic amines by gas-liquid chromatography *J pharm. Sci.* 1962 *51* 938–941

Brodie B.B K.F Finger P B Orlands, G.P Quinn and F Sulser Evidence that tranquillizing actio of reserpine is associated with change in brain serotonin and not in brain norepinephrine *J Pharmacol. exp Ther* 1960 *129* 250–256

Brooks, C.J W and E.C Horning Gas chromatographic studies of catecholamines, tryptamines, and other biological amines *Analyt Chem.* 1964 *36* 1540–1549

Brown, W.G L. Kaplan, A.R van Dyken and K.E Wirzbach Tritium as a tool for industrial and chemical research Int conf on the peaceful use of Atomic Energy I (Geneva 1955) Proc vol. 15 pp 16–23 New York 1956

Brownlee G and T L B Spriggs, Estimation of dopamine noradrenaline adrenaline and 5-hydroxytryptamine from single rat brains. *J Pharm. Pharmacol.* 1965 *17* 429–433

Callingham B.A. and R. Cass, A modification of the butanol extraction method for the fluorometric assay of catecholamines in biological materials, *J. Pharm. Pharmacol.* 1963 *15* 699–700

Callingham B.A. and D.F. Sharman The concentration of catecholamines in the brain of the domestic fowl (gallus domesticus) *Brit J Pharmacol* 1970 *40* 1–5

Capella, P and E.C. Horning Separation and identification of derivatives of biological amines by gas-liquid chromatography *Analyt Chem* 1966 *38* 316–321

Carruthers, M., P Taggart, N Conway D Bates and W Somerville Validity of plasma-catecholamine estimations. *Lancet* 1970 .. 62–67

Chang, C.C., A sensitive method for spectrophotofluorometric assay of catacholamines. *Int. J Neuropharmacol.* 1964 *3* 643–649

Clarke, D D S Wilk S.E. Gitlow and M.J Franklin Gas chromatographic determination of dopamine at the nanogram level. *J Gas Chromatog.* 1967 *5* 307–310

Cohen, Y J Bralet and W Janiec Etude par filtration sur gel de Sephadex G-25 de la liaison de l'adrénaline [14]C aux protéines seriques et tissulaires de Lapin. *C.R. Soc Biol. (Paris)* 1968 *162* 1320–1324

Crawford, T.B.B. and C.M Yates, A method for the estimation of the catecholamines and their metabolites in brain tissue *Brit J Pharmacol.* 1970 *38* 56–71

Cummins, L.M and M.J Fourier GLC determination of pseudoephedrine and related ephedrines in serum as the heptafluorobutyryl derivatives, gas chromatography electron capture detection. *Analyt Letters* 1969 403–409

Drell, W Separation of catecholamines from catechol acids by alumina. *Analyt. Biochem.* 1970 *34* 142–151

Ehrlen, I., Fluorometric determination of adrenaline, *Farm. Rev (Stockh.)* 1948 *47* 242–250

Engelman, K., B Portnoy and W Lovenberg, A sensitive and specific double isotope derivative method for the determination of catecholamines in biological specimens. *Amer J med Sci.* 1968 *255* 259–268

Engelman, K. and B. Portnoy A sensitive double isotope derivative assay for norepinephrine and epinephrine. *Circulat Res.* 1970 *26* 53–57

von Euler U.S and I Floding, A fluorometric micromethod for differential estimation of adrenaline and noradrenaline *Acta physiol. scand* 1955 *33* Suppl. 118 45–56

Fales, H.M and J.J Pisano, Gas chromatography of biologically important amines. *Analyt. Biochem.* 1962 *3* 337–342

First symposium on catecholamines, Bethesda 1958 *Pharmacol. Rev* 1959 *11* 233–566

Forrest, J.E. and R.A Heacock Thin-layer chromatography of some acetylated adrenaline derivatives. *J Chromatog.* 1969 *44* 638–640

Forrest J.E., R.A. Heacock D.J Roberts and T.P Forrest The nature of some solvent-derived artifact spots observed during the chromatography of adrenaline *J Chromatog* 1970 *51* 525–533

Franklin, M.J and J Mayer Advantages of the double isotope approach to catecholamine analyses. *Atomlight New England Nuclear Corp* 1968 *67* 1–6

Franklin, M.J and J Mayer Personal communication 1969

Frère J.M and W.G Verley O the use f an acetylated derivative of normetanephrine for gas chromatographic analysis. *J Chromatog* 1967 *30* 397–404

Goldstein, M A.J Friedhoff and C. Simmons, A method for the separation and estimation f catecholamines in urine *Experientia (Basel)* 1959 *15* 80–81

Goldstein, M., A.J Friedhoff C Simmond and N.N Prochoroff, Indirect evidence of synthesis of norepinephrine from 3-hydroxytyramine-1 [1] C in vivo *Proc. Soc exp. Biol Med.* 1960 *103* 137–140

O'Gorman, L.P., O Bomd J.A Kahn and L.R Gjesing, The metabolism of 1 3 4-dihydroxyphenylalanine in man *Clin. chim. Acta.* 1970 *29* 111–119

O'Hanlon, Jr J.F., H.C. Campuzano and S.M Horvath, A fluorometric assay for subnanogram concentrations of adrenaline and noradrenaline in plasma. *Analyt Biochem.* 1970 *34* 568–581

van Orden, L.S Quantitative histochemistry of biogenic amines. *Biochem. Pharmacol.* 1970 *19* 1105–1117

Paasonen, M.K. and P.B Dews, Effect of raunescine and isoraunescine on behaviour and on the 5-hydroxytryptamine and noradrenaline contents of brain *Brit. J Pharmacol.* 1958 *13* 84–88

Parker K.D C.R Fontan and P.L. Kirk, Separation and identification of some sympatomimetic amines by gas chromatography *Analyt Chem* 1962 *34* 1345–1346

Piez K.A. and H Eagle, Systematic effect of [14]C-labeling on ion-exchange chromatography of amino acids. *Science.* *1955 122 968–969*

Piez K.A and H Eagle, [14]C isotope effect on ion-exchange chromatography of amino acids. *J Amer chem. Soc.* 1956 *78* *5284–5287*

Rentzhog, L., Double isotope derivative assay of catecholamines. *Acta Pharmacol. (Kbh.)* 1970 *28* Suppl. 1 pp 74

Renzini, V., C.A. Brunori and C Valori, A sensitive and specific fluorometric method for determination of noradrenaline and adrenaline in human plasma. *Clin chim. Acta.* 1970 *30* 587–594

Ritzén, M Quantitative fluorescence microspectrophotometry of catecholamine-formaldehyde products. *Exp Cell. Res.* 1966 *44* 502–520

Roberts, D.J Chromatographic evidence for the formation as an artifact of an isoprenaline-like substance from adrenaline *Biochem. Pharmacol.* 1966 *15* 63–69

Saelens, J.K M.S Schoen and G.B Kovacsics, An enzyme assay for norepinephrine in brain tissue *Biochem. Pharmacol.* 1967 *16* 1043–1049

Saito H., S Woronkow and B Myers, A rapid and sensitive method for simultaneous determinatio of epinephrine and norepinephrine in human plasma. *J Circulat. J* *1969 33 677–683*

Sato T and V DeQuattro Enzymatic assay for 3 4-dihydroxymandelic acid (DOMA) in human urine plasma and tissues. *J Lab clin. Med.* 1969 *74* 672–681

Schroeter L.C T Higuchi and E.E. Schuler Degradation of epinephrine induced by bisulphite *J Amer pharm. Ass.* 1958 *47* 723–728

Second Symposium on Catecholamines, Milan 1965 *Pharmacol. Rev* 1966 *18* 1–804

Sekeris, C.E and P Herrlich Nachweis von N-Acetyldopamin bei einem Fall von Phaeochromocytom *Hoppe-Seylers Z physiol. Chem.* 1963 *331* 289–291

Sen N.P and P.L. McGeer Gas chromatography of phenolic and catecholic amines as the trimethylsilyl ethers. *Biochem. biophys. Res. Commun.* 1963 *13* 390–393

Shaw F.H The estimation of adrenaline. *Biochem. J* 1938 *32.* 19–25

Shepherd D.M and G.B West Effect of trichloroacetic acid on adrenaline chromatograms. *Nature (Lond.)* 1952 *4306* 797

Shore P.A and J.S Olin, Identification and chemical assay of noradrenaline in brain and other tissues. *J Pharmacol. exp Ther* 1958 *1 2* 295–300

Smith A and S.B Wortis, Formation and metabolism of N-acetyl-normetanephrine in rats treated with iproniazid *Fed. Proc* 1960 *19* 294

Smith, E.R.B and H Weil-Malherbe, Metanephrine and normetanephrine in human urine Method and results. *J Lab. clin Med.* 1962 *60* 212–223

Spedding, F.H J.E Powell and H.J Svec A laboratory method for separating nitrogen isotope by ion-exchange *J Amer chem. Soc* 1955 *77* 1393

Stern, J.S., M.J. Franklin and J. Mayer. Separation of epinephrine and norepinephrine on glass fiber paper. *J. Chromatog.* 1967 *30* 623–633
Temple, D.M. and R. Gillespie. Liquid ion-exchange extraction of some physiologically active amines. *Nature (Lond.)* 1966 *09* 714–715
Tyce, G.M. Metabolism of 3,4-dihydroxyphenylalanine by isolated perfused rat liver. *Biochem. Pharmacol.* 1971 *20* 3447–346
Vendsalu, A., Studies on adrenaline and noradrenaline in human plasma. *Acta physiol. scand.* 1960 *49* Suppl. 173
Vogt, M., The concentration of sympathin in different parts of the central nervous system under normal conditions and after the administration of drugs. *J. Physiol. (Lond.)* 1954 *1.3* 451–481
Waldi, D. Quantitative Bestimmung von Adrenalinlösungen mit Hilfe der d. nnschicht chromatographischen Vergleichsmetode. *Mitt. dtsch. pharm. Ges.* 1962 *32.* 125–128
Walle, T. Quantitative gas chromatography of picogram quantities of amines a 2,4-dinitrophenyl derivatives. *Acta pharm. Suec.* 1968 *5* 367–384
Weil-Malherbe, H. and A.D. Bone. The chemical estimation of adrenaline-like substances in blood. *Biochem. J.* 1952 *51* 311–318
Weil-Malherbe, H. The estimation of total (free + conjugated) catecholamines and some catecholamine metabolites in human urine. *Meth. biochem. Anal.* 1968 *16.* 293–326
Welsh, L.H., O^3 O^4 N-triacetyl-( )-epinephrine. *J. Amer. chem. Soc.* 1952. *74* 4967–4968.
Welsh, L.H. The analysis of solutions of epinephrine and norepinephrine. *J. Amer. pharm. Ass.* 1955 *44* 507–514
Whitehead, J.K. and H.G. Dean. The isotope derivative method in biochemical analysis. *Meth. biochem. Anal.* 1968 *16* 1–98

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 378

PERFUSION AND DIFFUSION IN SHOCK

A study of disturbed tissue-blood exchange in low flow states in canine skeletal muscle by a local clearance technique

BY
LENNART APPELGREN

GÖTEBORG 1972

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 378

From the Department of Surgery I University of Göteborg Sweden

PERFUSION AND DIFFUSION IN SHOCK

A study of disturbed tissue-blood exchange in low flow states in canine skeletal muscle by a local clearance technique

BY

LENNART APPELGREN

GÖTEBORG 1972

ORSTADIUS BOKTRYCKERI AKTIEBOLAG
GÖTEBORG 1972

Contents

R. TADIUS BOKTRYCKERI AKTIEBOLAG
GÖTEBORG 1972

The flow of material between the cell and blood also depends on the *position of the cell in relation to the framework of exchange vessels* and the *distribution of flow* within them maldistribution of flow may change this relation and so the material flow

The interstitial space

Diffusion The interstitium amounts to 13—20% in skeletal muscle (e.g. Agnew 1965) but little is known of its detailed function as a transport medium for diffusion. Conclusions from macroscopic measurements of steady state diffusion of extracellular hydrophilic tracers into macroscopic tissue cylinders or through macroscopic plane slices of tissue (Mc Lennan 1957 Page and Bernstein 1963 Brookes and Mackay 1971) cannot be simply transferred to a microscopic scale because of difficulties in transforming the geometrical formalism of steady state diffusion at the macroscopic level to a non-steady state diffusion at the microscopic level.

The prevailing opinion is that *free diffusion* of hydrophilic electrolytes and non-electrolytes through the interstitial space, modified by *geometrical* factors, can explain transport through the interstitial space. Thus the reduced tissue diffusion is interpreted as due to a reduction of available area for diffusion (to approximately 17% in skeletal muscle) and to an increase of the diffusion distances (factor 1.5—4) because of tortuosity of the diffusion pathways (Mc Lennan 1957 Brookes and Mackay 1971) In special cases such as with the specific geometry of the extracellular space of the crystalline lens the geometrical conditions may lead to anisotropic diffusion (Paterson and Maurice 1971) The geometry of the interstitial space in skeletal muscle also suggests anisotropy of diffusion (cf Table 1 p 14 and Fåhreus 1955)

The existence of dead end pores, more slowly accessible to extracellular tracers (Page *et al* 1964 Birks and Davey 1969) does not influence steady state diffusion through a sheet of tissue but their effect at the microscopic level is difficult to estimate.

It should be noted that the "exclusion volume" effect and the "sieve" effect of hyaluronic acid of the interstitium (e.g. Day 195., Ogston and Sherman 1961 Laurent 1970) and the diffusion delay by chemical interaction with the polymer network of the interstitium (e.g. Joseph *et al* 1952) are included in the formally calculated geometrical factors of macroscopic diffusion.

The cell membrane prevents significant access of predominantly extracellular hydrophilic substances such as iodide to the *intracellular space* (e.g. Wallace and Brodie 1937) Iodide can be rapidly and reversibly washed out from the muscle *in vitro* (Hill and MacPherson 1954) Within the cytoplasm of muscle cells the diffusion is, as far as is known 2—3 times slower than extracellularly with calcium ion as a remarkable exception (Kuschmer et al 1971) and there is no difference in intracellular diffusion between electrolytes and non-electrolytes.

Shock may change the distribution of ions and fluid between the cell and the interstitium (Shires and Carrico 1966, Hagberg *et al* 1967 Cunningham Shires and Wagner 1971) The extent to which this influences the study of transport of an extracellular anion such as iodide from the outside of the cell to blood is unknown but is clearly a critical question in the interpretation of the present results. The study of the equilibration of iodide between muscle and plasma in shock 28—30 might give some indication as to the extent of such a redistribution

The importance of changes in the physico-chemical and geometrical characteristics of the interstitial space for diffusion in shock is little known (Koven *et al* 1970 Fulton 1971 *Cf* Haljamae 1969 1970 for discussion) However steady state diffusion through tissue slices *in vitro* seem relatively insensitive to the time from the transfer of the tissue from a perfused state in the animal to the artificial medium where the measurements are done (Brookes and Mackay 1971 Paterson and Maurice 1971) The electric resistance of skeletal muscle which is related to the mobility of ions within the interstitial space (as diffusion) is decreased markedly when the muscle is made anoxic. This can be due to an increased mobility but may also be explained by the hyperosmosis of the interstitial space induced by anoxia (Fåhreus 1955)

The current concept is that *lipophilic* solutes such as xenon diffuse freely through tissue as a homogeneous isotropic medium with no difference between intracellular and extracellular space (Kety 1951 Perl 1962, Lassen 1967 a) This concept has been questioned by Hills (1967)

Perfusion The role of fluid movement between tissue and blood in interstitial space transport of low-molecular substances is not clear (Lundgren and Mellander 1967 Cf Rapaport *et al* 1952, Landis and Pappenheimer 1963) The convective transport within the interstitial space would be of the order 0 3 ml/min/100 g muscle or 1 5 ml/min/100 ml interstitial space or $\frac{1.5 \times 100}{d}$/ml/min/100 ml fluid phase of the interstitial space, where d may be anywhere between 10% and 90% (Gersh and Catchpole 1960 *Cf* comments to table 1 p 15)

The capillary wall

Diffusion *Lipophilic* substances diffuse freely across the endothelial cells of the *capillary wall* (Renkin 1952, Landis and Pappenheimer 1963) while *hydrophilic* low molecular substances pass by diffusion through a small fraction of the capillary wall, through pores or slits 35—100 Å wide (Pappenheimer and Soto-Rivera 1948 Pappenheimer Renkin and Borrero 1951 Pappenheimer 1953 Grotte 1956, Landis and Pappenheimer 1963 Renkin 1964 Alvarez and Yudilevich 1969 Crone and Lassen 1970) For morphological aspects of the capillary wall in skeletal muscle, see Karnovsky (1968 1971)

The capacity of the capillary bed for diffusion of low molecular substances and for hydrodynamic fluid exchange seems little changed in shock and ischemia (Mellander and Lewis 1963 Arturson and Thorén 1964) and even in a second degree burn area (Artursson and Mellander 1964) If anything a low flow state with anoxia, cell damage and an "inflammatory reaction" (Eriksson, personal communication) with release of substances such as proteases and kinins would in crease capillary permeability an old concept in shock (see Wiggers 1950 for references)

"*Perfusion*" Flow across the capillary wall is of the order of 0.3 ml/min/100 g tissue. This is one order of magnitude less than the diffusional transport across the capillary wall and need not be considered further (see Landis and Pappen heimer 1963 and Table 1)

The vascular bed

Morphology The vessel distribution in skeletal muscle is known in detail e.g. Spalteholz 1888 Krogh 1919 Illig 1961 Wiedeman 1963 Hammersen 1968 1970 Smaje, Zweifach and Intaglietta 1970, Eriksson and Myrhage 1972 a Cf Hammersen 1968 and 1970 for a critical discussion of previous work) but it is understood that there may be variations of the pattern on a microscopic scale (over some 10 µ) in a tissue considered as homogeneous on a macroscopic scale.

Since a detailed morphological information cannot be utilized fully here it may be sufficient to state that the general course of exchange vessels in skeletal muscle is parallel to muscle fibres (Fig. 1) and that the exchange area with an open capillary network has been estimated to 0 7 (Pappenheimer *et al* 1951) to 0 9 m^2/100 g muscle (Eriksson and Myrhage 1972 a) As the present study is concerned with several mixed muscles of the hindleg of the dog a review of the morphological and functional differences between red white and mixed muscles will not be given.

The feeding arteries and veins to a muscle bundle of about 1500 fibres (as in the tenuissimus muscle in the cat, Eriksson and Myrhage 1972 a) enter the muscle transversely and close together and lead to a central artery (CA) and vein (CV Fig 1) which run parallel to the fibres and close to each other (distance 50—200 µ) From the central artery and vein transverse endarterioles (TA) and end venules (TV) run transversely or obliquely across the fibres at a distance between 10 and 1000 µ (Hammersen 1968 Eriksson and Myrhage 1972 a) The trans verse endarterioles and endvenules divide into successively smaller branches and finally subdivide into capillaries. This pattern seems to be largely the same in several skeletal muscle of different species (Illig *loc cit* Hammersen *loc cit* Eriksson, personal communication)

The concept of preferential channels, that is of short wide bore capillaries with favourable pressure gradients in low flow states is old (Langley 1911 Cf Zwei fach and Metz 1955) but recent studies have not confirmed such channels mor



Figure 1 The terminal vascular bed of a skeletal muscle (the tenuissimus muscle of the cat) The central artery (CA) and vein (CV) run parallel to the muscle fibres. E darterioles (TA) and endvenules (TV) branch off transversely or obliquely to the central vessels. The capillaries run parallel to th muscle fibres and have numerous cross connections. Most of the vessels (but not all) are filled with contrast. The discontinuity of some f the capillaries is due to their running out of focus. The funnel shape of the entrance of the transverse venules into the central vein, which is not the normal appearance *in vivo* may be due to an increased venous pressure. Indian ink perfusion of vasodilated muscle (papaverine) Cleared according to Spalteholz (1888), × 50
(Reproduced by courtesy f Drs. Elof Eriksson and Rhagnar Myrhage Laboratory of Experimental Biology/Chief Professor P I. Brånemark/Department f Anatomy University of Göteborg)

phologically in skeletal muscle they do appear within the muscle fascia (Eriksson and Myrhage 1972 a. *Cf* Hammersen 1968 for a critical discussion of previous works) Furthermore, there is no morphological evidence for a separation of skeletal muscle blood flow into a 100% efficient nutritive component and a 100% non-nutritive shunt component (cf Friedman 1965 1971) However connective tissue septa within the muscle (Barlow Haigh and Walder 1961 Walder 1968) as well as other tissues than muscle such as bone marrow within a skinned hindleg preparation have a different circulation than muscle tissue (Renkin 1971 *Cf* Renkin 1955)

It is well known that small arterioles and venules are subject to active as well as passive variations of lumen. These may be of importance for the facilitation of flow by increase of shear rate brought about by decreased vessel diameters at low flows (Djojosugito *et al* 1970 Baeckström *et al* 1971 a) However there is no complete collapse of capillaries at low intraluminal pressure (cf Nicholl *et al* 1953 Eriksson and Lisander 1972 b) The capillaries behave as holes bored in a massive tissue (cf Landis 1971) Distal small arterioles close to the capillaries are more sensitive to local vasodilating influence opposing vasoconstriction than are venules, proximal arterioles and small arteries (Lewis and Mellander 1963

Mellander and Lewis 1963 Folkow *et al* 1971 Eriksson and Lisander 1972 b)

Perfusion The variation in blood flow and flow distribution by vasoactive changes in vessel dimensions and by changes in perfusion pressure are known to a large extent as spatial averages measured over a relatively large muscle mass (0.5—100 g· see Mellander and Johansson 1968 Mellander 1971) The variation of perfusion between different small areas of skeletal muscle (200—1000 mg) is small in the dog gastrocnemius muscle perfused at different flow rates (Thompson *et al* 1959) and between areas (200—500 mg) injected or atraumatically labelled with [133]Xenon in the same hyperemic muscle in the cat (Tönnesen and Sejrsen 1970) However the degree of perfusion heterogenicity in hyperemia between a 10-fold smaller tissue areas (20—200 mg) increased from an amazing homogeneity in the non isolated muscle to between 0 and 250% of mean perfusion in surgically isolated muscle (Paradise, Swayze, Shin and Fox 1971 cf Moore and Baker 1971) Very small volumes of saline injectate of [133]Xenon (1—10 µl) showed the same variation in isolated hyperemic muscle (Tönnesen and Sejrsen 1970).

Flow distribution At a microscopic level there is an uneven but generally concurrent flow in capillaries in the muscle at rest (Eriksson *et al* 1972 a, b) while with hyperemia at normal pressure capillary flow becomes faster and more even (Johnson 1970) The microcirculatory flow pattern has been studied in detail by vital microscopy in several muscles of different species (Zweifach and Metz 1955 Grant 1964 Johnson 1970 Smaje, Zweifach and Intaglietta 1970 Eriksson and Myrhage 1972 a) also at anoxia, at low perfusion pressure and in shock (*Cardon et al 1971 Eriksson and Lisander 1972 b, see also Ditzel and Lewis* 1971 for several ref) The general impression is an increasing inhomogeneity of the microcirculation in skeletal muscle in shock.

The microcirculation in shock has been studied extensively for several decades and in different tissues easily accessible to microscopic investigation (see e.g Gelin 1956 Zweifach 1958 Knisely 1963 Wells 1964 Berman and Fulton 1965 Shepro 1968 Nicoll and Frayser 1967 Matheson 1969 Malinin Linn, Callahan and Warren 1971) However the complex interplay between on one hand the properties of the physical constituents of blood and the blood rheology *in vitro* and on the other hand the vessel diametres, the changing pressure gradients, the flow and the flow distribution is still imperfectly understood (Chien 1969 Chien *et al* 1971 Weed and Lacelle 1969 Dintenfass 1971) Recent studies (Djojosugito *et al* 1970 Baeckström *et al* 1971 1971 a, Eriksson *et al* 1972 a, b) have filled in some of the gaps of quantitative knowledge on the relation between rheological properties of blood and skeletal muscle circulation under normal conditions (rest, vasoconstriction and hyperemia) at low perfusion pressures and in hemorrhagic shock.

The current concept is that the distribution of flow through capillaries is regulated substantially separately from flow and by precapillary "sphincters" at the

arterial entrance to the capillaries (Chambers and Zweifach 1944 Zweifach and Metz 1955 cf Mellander and Johansson 1968 Mellander 1971) as early as 1919 (a) Krogh stated that "the arteriomotor and capillariomotor systems were able to act in opposite directions" This concept has provided a fruitful basis for detailed functional analyses of the skeletal muscle circulation (e.g. Mellander *et al* 1968 1971 cf Winbury 1971)

However recent studies by vital microscopy do not confirm the existence of a precapillary sphincter activity in skeletal muscle (Johnson 1970 Eriksson *et al* 1972 a, b) These studies rather indicate a random variation of capillary flow where changing pressure gradients and the small deformation drag of blood corpuscles at the capillary entrance (Rand and Burton 1964 Weed and Lacelle 1969 Schmidt Schoenbein 1970 Dintenfass 1971 Chien *et al* 1971) change the flow intermittently (Johnson 1970 *cf* Cardon *et al* 1971) With this mechanism the regulation of the degree of perfusion of capillaries becomes partly parallel with regulation of flow and would occur upstream from the capillaries by flow pressure regulation at the level of small arterioles, acting upon groups of capillaries (*cf* Algire and Mervine 1955)

Such a mechanism should be sensitive to changes in the corpuscular elements in shock (Weed and Lacelle 1969 Ljungquist 1970 Dintenfass 1971 Chien *et al* 1971 Goldstone *et al* 1970 Schmidt Schoenbein 1971 *Cf* Discussion pp 48—49) with an early *instability* (at maximal sympathetic activity opposing local vasodilation) of number of *groups* with many well perfused capillaries and a later *decrease* of number of perfused capillaries On the contrary a vasomotor regulation at the level of the capillary entrance would react by an early *decrease* of very short duration followed by a sustained *increase* of number of open capillaries with the accumulation of local metabolites (Cobbold *et al* 1964 Lewis and Mellander 1962 Mellander and Lewis 1963 Renkin 1968)

For completeness, the "regulation" on the off flow side (by stagnation in venules) must be considered, especially in low flow states and with conditions of disturbed blood rheology as with increased erythrocyte aggregation and sludging (Knisely and Bloch 1942 Gelin 1956, 1963 Djojosugito *et al* 1970) *Hysteresis phenomena* with stagnation of flow within capillaries or in venules might be more accented in a low perfusion state with low perfusion pressure, low shear rates and altered blood microrheology

Thus interaction between many different variables of the control of capillary flow and flow distribution the delicate differentials of the perfusion pressures and transmural pressures of each vascular section, nervous, myogenic, chemical and passive changes of vessel diameters and the rheology of blood and blood corpuscles in relation to different vessels (including hysteresis phenomena) will give many possible response patterns, even within the same vascular bed and during normal conditions As discussed above, one of the weakest links of this complex control system would seem to be the interaction between perfusion pressure geometry of the vascular bed and blood microrheology

Theoretical considerations on tissue to blood transport

Elementary steps of transport It should be appreciated that much of the information in this condensed review on the system for passive transport in skeletal muscle cannot be used in more than a qualitative sense in a discussion of the rate of tissue to blood exchange. However an approximate magnitude of perfusion and diffusion in skeletal muscle of the two isotopes used in this study (^{131}Iodide and ^{133}Xenon) may be calculated and used for an estimation of the significance and interaction of the different elementary steps of transport. The estimations and considerations given below rely heavily on several comprehensive treatments such as Kety (1951) Landis and Pappenheimer (1963) Perl (1963) Renkin (1969) and Crone and Larsen (1970).

In *Table 1* perfusion is given in ml/min/100 g or $\frac{\text{mol/min/100 g}}{\text{mol/ml}}$ while diffusion is calculated as permeability-surface area products PS $= \alpha \cdot D \cdot A \cdot 60/\beta \cdot \Delta x$ ml/min/100 g or $\frac{\text{mol/min/100 g}}{\text{mol/ml}}$ according to Fick's first law for steady state diffusion in one dimension (Fick 1855) The symbols used are:

- α, geometry factor for reduction of area for diffusion
- D free diffusion coefficient in cm^2/sec
- A, surface area for diffusion in cm^2/100 g
- β tortuosity factor
- Δx and Δy diffusion distances in cm (transverse and longitudinal to vessels)

For rough estimations a concentration gradient 1 to 0 9 (which reduces diffusion 10 times compared to a concentration gradient 1 to 0) may be equalized with homogeneity

It is tentatively suggested that perfusion determines transport of *xenon* at hyperemia but that some attenuation by longitudinal gradients may occur Attenuation by vein to artery diffusion (Aukland, Akre and Leraand 1967 Tönnesen and Sejrsen 1970 Tönnesen 1969 Sejrsen 1970) is suggested to be less than 10% at hyperemia but becomes increasingly important at lower flows. However vein to artery and vein to tissue diffusion recruits new tissue areas for labelling with xenon. These areas are perfused at the same flow level as the rest of the muscle and are of course limited in their extent because of diffusion distances and regionality of perfusion. An hypothesis worth an experimental test, which would be of considerable practical interest (see e.g Jansson 1969) is therefore whether at low flows xenon transport becomes again solely dependent on perfusion This hypothesis has been tested in a small study comparing xenon clearence from a local depeau and venous outflow (pp 30—33)

It is further suggested that *iodide* transport is determined by the diffusion through the capillary wall both at hyperemia and in the higher range of resting flows while generally both perfusion and diffusion are significant at lower rest

Table 1 Approximate perfusion and d ff sion magnitudes in 100 g skeletal muscle (dimensions, see text).

	Iodide	Reference	Xenon	Reference
Vascula perfusion				Mellander et l 1968, Mellander 1971
rest	3—10			
hyperemia	20—60			
vasoconstriction	0.5—4			
Extra ascular perfusion				Mellander 1971 cf Landis and Pappenheimer 1963
capillary wall	0.3—0.8			
interstitial	0.3—0 8?			
lymph flow	low			
Diffusion capil lary wall:				
D	~6 × 10⁻⁵	Brookes et al 1971 cf Hill and Mac Pherson 1954 Pater son t al 1971	0.65 × 10⁻⁴	Sejrsen et al 1968 Tönnesen 1969 cf Hills 1967
α	0.0001?)		1	
Δ x	10^{-4}		10^{-4}	
β	1		1	
A, hyperemia	0.9 10⁴	Eriksson t al 1972 a	as iodid	
rest	0.3 × 10⁴			
PS hyperemia	14		6000	
rest	4.7		2000	
Diffusion intersti tium, transverse				
D	see capillary wall			
α	0.17	Brookes et + 1971		
β	3 7			
A	see capillary wall			
Δ x, hyperemia	2 10⁻⁴ ?)	Eriksson it		
rest	] 3 ×			
PS, hyperemia	320 (1070?))			
rest	62 (400?))			

	Iodide	Reference	Xenon	Reference
Diffusion interstitium, longitudinal:				
D	see capillary wall			
α	0.17	Brookes et l 1971	1 ?	
β	1 ?4)		1 ?	
A	0.2 of transverse A)		as iodide	
Δx	4×10^{-4}		as iodide	
PS	12		18	
Diffusion vei to artery (counter current) and ein to tissue				
D	see capillary wall			
α	see capillary wall; low?		1	
β	1 ?		1	
A	1706)		as iodide	
Δx	1.5×10^{-2}		as iodide	
PS	low?		4.5	

1) 0.1 of the estimation 0.1 / by Pappenheimer (1963; cf Kedem and Katchalsky 1958 Alvarez and Yudilevich 1969 Karnowsky 1970, P ppenheimer 1970 Trap-Jensen and Lassen 1970) Cf PS by tracer kinetic methods (e g. Renkin 1959 Yudilevich t al 1968, Trap-Jensen t al, 1970).
2) Muscle cell diameter 40μ (Eriksson and Myrhage 1972 a).
3) Cylindrical geometry and steady state diffusion (cylinder diameter 46μ, capillary diameter 6μ).
4) Larger than 1 ($3.7 \times \frac{2r}{\pi r} \simeq 2$) ? Cf Introduction, pp. 7 interpretation of geometrical factors in tissue diffusion.
5) Capillary length 400μ.
6) Coarsely estimated from data of the tenuissimus muscle in ca (Eriksson and Myrhage 1972) Distance between parallell artery and vein 150μ. Area for counter-current diffusion in a muscle, 2,5 cm long and with diameters 0.4 × 0.04 cm (breadth 2 × essel diameter 100μ) × (length of close contact between artery and vein 2.7 cm)

ing flows. *Longitudinal diffusion of iodide must be seriously considered* (cf Crone and Garlick 1970 Bassingthwaighte, Knopp an Hazelrig 1970)

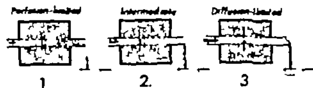
The perfusion of the interstitial space and capillary wall may have been underestimated here to an unknown degree (cf p 8) while longitudinal diffusion might have been overestimated (*cf* note 4 to table 1) A perfusion value 0 1—0.3 seems low compared with PS 5 for iodide through the capillary wall and with PS 12 longitudinally through the interstitium (6 ? cf Table 1 note 4) even if concentration gradients as low as 0 1 are assumed. Paracapillary circulation in the classical sense of Starling (1896, *cf* Landis and Pappenheimer 1963) may be much larger or more likely efficient within a much smaller volume within the interstitial space (p 8) and so may significantly counteract the impeding effect of longitudinal gradients. This may be one explanation of several possible for the augmentation of tissue-blood exchange by increased fluid exchange between tissue and capillary (Lundgren and Mellander 1967)

Thus the main barrier for transport seems to be localized at the capillary wall (iodide) or the venous exit (xenon) transverse relaxation times [7]) within the tissue cylinder (iodide) or tissue cylinder + capillary (xenon) should be short and are estimated to be of the order of 0.1—10 sec (Kety 1951 *cf* Johnson 1970) Experimentally the times for establishing concentration gradients (longitudinal?) within the interstitial space and between the interstitium and the capillary *within the tissue cylinder* are of the order 1 minute (Crone and Garlick 1970 Ussing 1953) The relaxation times within the whole tissue will be much longer if the effects of inhomogeneous perfusion and longer diffusion distances are considered (Crone and Garlick 1970, Lassen and Sejrsen 1971)

These data will be referred to more fully in the discussion of experimental results (pp. 00—00). At this stage one may conclude that there are *least marginals* for transport disturbances in the *vascular perfusion* (xenon and iodide) and the *diffusion through the capillary wall* (iodide) but that a 10-fold decrease of $A/\Delta x$ by inhomogeneity of flow in capillaries or a 10-fold decrease of $\alpha \; D'\beta$ in the interstitium may interfere with the transverse diffusion of iodide through the interstitium as well. *Longitudinal diffusion* (iodide, xenon), *extravascular perfusion* (iodide) and *blood to tissue diffusion* (xenon) are factors whose significance in shock will be impossible to estimate with gross calculations like these.

The tissue cylinder model Since Krogh and Erlang (1919) the integration of these elements of transport to a spatial average tissue-capillary cylinder model with different variation and refinements (Kety 1951 Sangren and Sheppard 1953 Renkin 1959 Crone 1963 Johnson and Wilson 1966 Bassingthwaighte *et al* 1970 Goresky 1963 1970) has been used in the interpretation of experimental data on the material balance of nutritives and metabolites in an organ, e.g of oxygen (e.g Krogh 1919 Kety 1951 1957 Wright and Sonnenschein 1961 Lübbers *et al* 1968 Honig *et al* 1971 *Cf* Landis and Pappenheimer 1963)

7) I.e. the time taken to reduce the difference between initial conditions and steady state to 1 e.



Q C
Q C
Q→C PS
CLEARANCE % X 100
C
Q
2
3
PS
Q BLOOD FLOW

Figure 2. A schematic illustration of the tissue clearance concept. Upper panel Flow (Q) is symbolized by the volume of fluid in the left graded cylinder where the point density indicates the venous concentration. The volume of fluid in the graded cylinder to the right indicates clearance (C) here the point density is the same as in the tissue. From 1 to 3 increasing flow rates (and lines flow rates)

as well as in the interpretation of experimental data on tracer kinetics (e.g. Kety 1951 Renkin 1959 Gosselin *et al* 1970 a, 71) As to tissue oxygenation the model provides means of predicting place and level of the critical concentration of oxygen in the tissue with variation of flow hematocrit, oxygenation of arterial blood, inter-capillary distance and tissue oxygen consumption and so *realizes the mutual interaction between metabolism and transport* (p 6) in a very detailed model within the limits of the tissue cylinder model (Knisely *et al* 1969)

Exclusion of cell metabolism (as with inert tracers) and assumption of a homogeneous extravascular compartment (cf Comments to Table 1) give a model in which the relative magnitudes of PS of the capillary wall and flow (Q) determine the transport. It is convenient to express transport in relation to Q as extraction E (which is the fractional saturation of venous blood relative to the mean tissue concentration) Clearance C_i of a substance i (Renkin 1955 1969) can be defined by

$$C_i = E_i \times Q \quad (1)$$

For tissue to blood transport tissue clearance may be described as the volume of blood in equilibrium with the tissue holding the same amount of tracer as the venous outflow (Fig. 2) The simplified tissue cylinder model gives the following relation between extraction and flow (Kety 1951 Renkin 1955 1959 Crone 1963 Johnson and Wilson 1966)

$$E_i = C_i/Q = 1 - \exp(-PS_i/Q) \quad (2)$$

ing flows *Longitudinal diffusion of iodide must be seriously considered* (cf Crone and Garlick 1970 Bassingthwaighte, Knopp an Hazelrig 1970)

The perfusion of the interstitial space and capillary wall may have been underestimated here to an unknown degree (cf p 8) while longitudinal diffusion might have been overestimated (*cf* note 4 to table 1) A perfusion value 0 1—0 3 seems low compared with PS 5 for iodide through the capillary wall and with PS 12 longitudinally through the interstitium (6 ? cf Table 1 note 4) even if concentration gradients as low as 0 1 are assumed. Paracapillary circulation in the classical sense of Starling (1896, *cf* Landis and Pappenheimer 1963) may be much larger or more likely efficient within a much smaller volume within the interstitial space (p 8) and so may significantly counteract the impeding effect of longitudinal gradients. This may be one explanation of several possible for the augmentation of tissue-blood exchange by increased fluid exchange between tissue and capillary (Lundgren and Mellander 1967)

Thus the main barrier for transport seems to be localized at the capillary wall (iodide) or the venous exit (xenon) transverse relaxation times [7]) within the tissue cylinder (iodide) or tissue cylinder + capillary (xenon) should be short and are estimated to be of the order of 0 1—10 sec (Kety 1951 *cf* Johnson 1970) Experimentally the times for establishing concentration gradients (longitudinal?) within the interstitial space and between the interstitium and the capillary *within the tissue cylinder* are of the order 1 minute (Crone and Garlick 1970 Ussing 1953) The relaxation times within the whole tissue will be much longer if the effects of inhomogeneous perfusion and longer diffusion distances are considered (Crone and Garlick 1970 Lassen and Sejrsen 1971)

These data will be referred to more fully in the discussion of experimental results (pp 00—00) At this stage one may conclude that there are *least marginals* for transport disturbances in the *vascular perfusion* (xenon and iodide) and the *diffusion through the capillary wall* (iodide) but that a 10-fold decrease of $A/\Delta x$ by inhomogeneity of flow in capillaries or a 10-fold decrease of α D/β in the interstitium may interfere with the transverse diffusion of iodide through the interstitium as well. *Longitudinal diffusion* (iodide, xenon) *extravascular perfusion* (iodide) and *blood to tissue diffusion* (xenon) are factors whose significance in shock will be impossible to estimate with gross calculations like these.

The tissue cylinder model Since Krogh and Erlang (1919) the integration of these elements of transport to a spatial average tissue-capillary cylinder model with different variation and refinements (Kety 1951 Sangren and Sheppard 1953 Renkin 1959 Crone 1963 Johnson and Wilson 1966 Bassingthwaighte *et al* 1970 Goresky 1963 1970) has been used in the interpretation of experimental data on the material balance of nutritives and metabolites in an organ, e.g of oxygen (e.g Krogh 1919 Kety 1951 1957 Wright and Sonnenschein 1961 Lübbers *et al* 1968 Honig *et al* 1971 *Cf* Landis and Pappenheimer 1963)

7) I.e. the time taken to reduce the difference between initial conditions and steady state to 1/e.

The calculation of the local tissue clearance

$$C_i \text{ ml/min/100 g} = k_i \text{ min}^{-1} \times \lambda_i \text{ ml/g} \times 100 \text{ g} \quad (3)$$

where k_i is the fractional disappearance rate[a]) from a local depeau in the tissue and λ_i the partition coefficient between tissue and blood, is completely general Renkin 1955 1959 Lassen and Trap-Jensen 1968) This is the case whenever k_i is estimated after "uniform labelling by local injection (Lassen 1967) However from the discussion above it follows that there may be several interpretations as to why a clearance value is less than flow

A time dependent turnover of tracer can *a priori* be expected in a complex nonhomogeneous biological system, even in a steady state with respect to exchange of nutritives and metabolites (Bergner 1962) This is true except under such limiting conditions (Bergner 1961 Lassen and Sejrsen 1971) which cannot be utilized in practice. As will be further elaborated in the discussion (pp 44—45), the relation between the dynamics of transport of nutritives and metabolites and data of tracer kinetic studies are not simple the results of tracer kinetic studies are therefore expected to be limited applicability This is especially the case if the lumping effect of lack of experimental precision is considered (Bergner 1962)

The "double isotope" local tissue clearance method

The local tissue clearance technique for the estimation of capillary permeability of small extracellular hydrophilic ions with the simultaneous use of an inert gas, ^{133}Xenon for the estimation of flow was first described by Lassen (1964 1967 a) Lassen intended the method primarily for clinical estimations of the factor capillary permeability (P) of the maximal capillary diffusion capacity (PS) of hydrophilic substances in hyperemic skeletal muscle under conditions in which xenon clearance was several times higher than PS of the ion and the ion extraction low In this situation there is only a small correction for backdiffusion into tissue or expressed in another way a small interference between diffusion and perfusion. Other authors have used the method for *a general description of transport conditions of small hydrophilic ions such as iodide within the whole physiological range of skeletal muscle circulation* (Gosselin *et al* 1967 1970 1970 a 71 Appelgren and Lewis 1967 1968 1970) in the same sense that the analyses of blood tissue equilibration and blood to tissue clearances of $^{42}K^+$ or $^{86}Rb^+$ were used by Renkin (1955 1959 1962 a, b c) There is no *a priori* reason to assume that the barrier function of the capillary wall (capillary permeability) is studied with this method instead PS would indicate an overall perme-

a) k_i may be looked upon as *time-dependent* turnover function of the residual tracer within the field of the detector (cf Appendix I pp. 58—60) The term clearance constant used in some of the original papers (I II III) is therefore not wholly adequate.

Comments on material and methods

Eighty-six adult male and non-pregnant female *dogs* weighing between 15 and 35 kg were used in the study Where nothing else is stated the dogs had the spleen intact All dogs also had intact adrenals. The dogs were not heparinized unless otherwise stated.

General methods

The animals were *anaesthetized* with intra enous thiomebumal sodium (Penthotal®) with repeated small doses as needed throughout the experiment. The anaesthesia was kept as shallow as possible to prevent shivering and moving during registration of radioactiv ty at an injection site The dogs breathed spontaneously through an endotracheal tube

During the experiments the animals lay supine and covered by a blanket. The rectal *temperature* was kept between 38 and 39° C by adjusting the blanket and by external heating.

Short, wide bore *catheters* were inserted into the right carotid artery for recording of blood pressure and periodic sampling f arterial blood and into the right jugular vein for slow infusion f saline (1.8—2.0 ml/kg bwt/hou) or for infusion of dextrans or blood.

Arterial blood pressure and arterio-venous pressure grad ents were measured by mercury manometers.

Venous outflow was measured with serial sampling into weighed heparinized glass tubes (enon learance vs venous outflow in isolated gastrocnemius muscle, pp. 30).

Arterial blood was sampled into heparinized glass tubes for

hematocrit (microhematocrit, no correction for trapped plasma)

radioactive measurements f blood and plasma samples (partition coefficient of iod de, pp 28—30).

Arterial blood (III) for *lactate and pyruvate* (Biochimica Test No. 15972 and 15973 C.F Boehringer & Sohne, Mannheim, Germany) was sampled in 0.6 M perchloric acid and the meta bolites determined spectrophotometrically according to the original description of the manufacturers.

Muscle biopsies (0.2—0.8 g) were taken in duplicate from the muscles used for clearance measurements.

Radioactivity of blood plasma and muscle samples was measured in well-counter by stan dard gamma-counting procedure.

Dextrans The use of dextrans as plasma volume expanders and as flow improving and as flow disturbing gents has been reviewed from several clinical and experimental aspects (e.g. Gelin 1956, H t 1968 The Scientific and Medical Staff of Pharmacia AB 1968 Gruber 1968 Ingelmann, Grönw ll, Gelin and Eliasson 1969) The two dextran fractions used in this study[9]) were *dextran 40* (Rheomacrodex®) $\overline{M}_w$ 40.000; called low molecular weight dextran or LMWD in I—VI) given in 10% solution n saline, and *dextran 1000* (an experimental high molecular weight dextran fraction (HMWD in III and VI), $\overline{M}$ 1.000.000) given in 15% solution.

Blood for transfusion (VI) was collected in 20% standard ACD-solution and stored sterile at 4 C.

Vasodilation was used with the intention of expanding the magnitudes of clearance and PS; of resting skeletal muscle (I—III) and in the more detailed study of the passi e vascular bed

9) Kindly supplied by Pharmacia AB, Uppsala, Sweden.

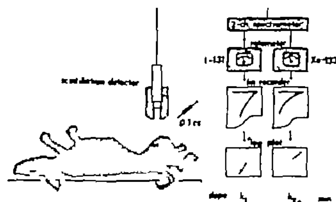


Figure 3 Experimental set up for determination of local ^{131}Iodide and ^{133}Xenon clea rances after injection of 0.1 ml saline solution of the isotopes into a muscle of the hindleg of the dog. For details, see text to (I) and (III).

in shock (IV—VI). Vasodilation was generally achieved by papaverine 0.8 mg/ml in the injec tate. This concentration was found to give maximal increase f Q_X and PS_I from control to values comparable to vasodilation by ischemia + muscular work (V). The dose response of

locally pplied papaverine may be summarized as follows

papaverine in the injectate, mg/ml	0	0.4	0.8	1.6	3.2
Q_X /s of control	100	300	500	300	200
PS_I / of control	100	150	200—300	70	70

Constant slopes of iodide and xenon activities in semilogarithmic diagram dow to 20 to 40% of initial activity indicate reasonably constant washout conditions in spit f the probable inhomogeneity of asodilator concentration in the tissue (fig 9 11).

In 4 dogs (I) potassium ion (Kjellmer 1965 Skinner and Powell 1967) was used as a vasodilator in a concentration 20 mM in the injectate

Dose response

Potassium ion in the injectate, mM	0	6	15	20	30	50
Q_{Xe} / of control	100	130	170	220	250	100
PS_I / of control	100	140	180	220	200	90

I four dogs (V group B) vasodilation was chieved by muscular contraction f the calf muscles during ischemia. The sciatic nerve was exposed through small incision in the back of the thigh and by blunt dissection between the lateral and medial flexors f the knee. The peripheral *end of the severed nerve was stimul ted 5—10 minutes by bipolar silver electrodes (8* V square w ve pulses, 0.2 msec, 8/sec) during complete occlusion of the abdominal aorta.

Local tissue clearanc f enon and iodide Several practical (i jection technique, injectate volume, carrier curve evaluation) and theoretical (labelling f the tissue, curve evaluation) aspects are discussed in the introduction and the references given there (pp. 18—21).

Radioactive xenon, ^{133}Xe (pecific activity 2—10 Ci/cc X at s.t.p.)[10] in saline sol tion (w th and without vasodilator) was mixed with carrier-free sodium iodide ^{131}I[10]. 0.1 cm^3 was injected slowly via fine standard needle (outer diameter 0.4 mm) inserted through th skin into the belly f the muscle using the quadriceps, the gracilis and the gastrocnemius muscles, and for control purpose also the muscles of the foreleg, with random placement of injection sites. The injection needle was left in place for 30 sec. after completing the injection.

10) AB Atomenergi, Studsvik, Sweden.

The activities of residual ^{133}Xe and ^{131}I at the injection site were registered by a single scintillation detector (Fig. 3) *Fo details of radioactive measurements, see I and III.* The residual activities were registered linearly on a recorder and plotted subsequently logarithmically against time. From straight line segments, drawn by inspection, t½ were calculated. In general the first part of the curves (3—5 minutes at rest) were not used except with hyperemia. The method of evaluating the curves is illustrated in figures 8, 9 10, 11 and figure 1 (IV) and 1 (V). The rationale for this is discussed below

15—20 injections (depending upon the size of the animal) could be done in each animal without an unacceptabl increase of the background of ^{131}I

The *partion coefficient* is the equilibrium ratio of activity/g tissue to activity/g blood of the same hematocrit as that leaving the *field* of the detector Therefore the proper hematocrit is large vessel hematocrit.

The *partition coefficient* of xenon between muscle tissue and blood with correction for hematocrit as given by Andersen and Ladefoged (1967) has been used throughout the present study A discussion of the partition coefficient calculated for iodide is given in paper (I) and (V).

Throughout the study the entrance f iodide into erythrocytes d ring passage of blood through tissue has been disregarded (see (V) and p. 29).

The *double isot pe tiss clearance method* has been applied here *formal way* accepting

1) the possible injection artefacts,

2) the use of several mixed muscles of the hindleg,

3) the possible inclusion of connective tissue within the injection area,

4) the time dependence of the fractional disappearance rates of iodide (k_I) and enon (k_X),

5) *the lumping technique fo determining fractional disappeara ce rates (see below) and*

6) the pairing of k_I and k_{Xe}, registered simultaneously in time

as part of the *variation f experiment l data.* Some of these sources f scattering may be looked upon as *expressions f the nhomogeneity f the system and the non-st ady state cha acter f the washout process*

The isotopes ha e been assumed to be distributed to equ alent but not necessarily geometrically coinciding volumes within the tissue. The justification for the ddition of vasodilator to the injectat to induce hyperemi was given by a experimental test against hyperemia of the whole muscle (Paper V) Paired iodide and xenon clearances have been calculated from *simul taneously* registered fractional disappearance rates. These were calculated from t½ values estimated by lumping the semilogarithmic curves to straight line segments, o er periods 1—3 minutes (hyperemia) or 3—30 minutes (rest) (see figures 8 9 10, 11) The paired clearances have been plotted in an iodide clearance — xenon clearance diagram (paper I—IV Figures 13 14 p 37). It has been assumed that xenon clearance estimates flow within the distribution volumes of the isotopes. PS lues for iodide were calculated using formula (2) p. 17 *The finding of d fferent PS values or clearances of iodide at the same xenon clearance have been interpreted as d to different iodid transport in the cap llary bed at the same c pillary perf sion*

The distributing effect of tracer passage through the eins (cf Gosselin *et al* 1970) and of ein to tissue d ffusion within the *field of the detector* have been disregarded *and the different relations between iodide and xenon clearances interpreted as sole expression f changes in diff sion and perfusion within the c pillary bed* The possible underestimation of perfusion by xenon clearance in the flow range 1—15 ml/min/100 g has not been corrected for but has been considered in the discussion of the results. The extent of this underestimation has been studied experimentally in a small series of animals, comparing xenon clearance and venous out flow in isolated gastrocnemius muscle (pp. 30—33) using the same registration and calculation technique as described above

Calculation and formulas Details may be found in the introduction (pp 16—18) and in I, III and V

Residual acti ty in the local tissue depot by extern l gammaregistration

A_I relati e counts per minute

Fractional disappearance rate

$$k_I = \frac{-1}{A_I} \times \frac{dA_I}{dt}$$

$$k_I = \ln 2/(t^1/_2)_I$$

where $t^1/$ was estimated from straight line segments of the curves drawn by inspection.

Flow: $Q = \text{or} > C_I$

Clearance $C_I = k_I \times \lambda_I \times 100$ ml min/100 g ((3) p. 19)

$C_{X_e} = Q_{X_e} = k_X \times \lambda_X \times 100$ ml/min 100 g

Extraction $E_I = C_I/Q$ ((1), p. 17)

$E_I = 1 - \exp(-PS_I/Q)$ ((2) p 17)

Capillary diffusion capacity or permeability-surface area product of iodide

$PS_{WB} = -Q_X \times \ln(1 - C_I/Q_{X_e})$ ml blood min 100 g

$PS_p = PS_{WB} \times (1 - \text{hct})$ ml plasma min 100 g

Statistical evaluation Standard methods for calculation of mean, ariance, standard deviation, standard error of the mean, regression line and correlation coefficient ( ) were used Student t-test and the F-test were used for the test f null hypotheses such as $\bar{x} = \bar{y}$ $= 0$ and $s^2_1 = s^2_2$. However considering the difficulties met the interpretation of washout curves pp. 18, 24) and in grouping of iodide clearance-xenon learance values and PS-values (Cf V) for statistical ev luation, the clearances and PS-values ha in general been presented directly in the diagrams nd the range within which they were found indicated by shaded areas (I—VI). A consequent eval ation f the difference in position of these areas in control and shock has not been done. Further comments on this are gi en on p. 37

Experimental procedures

The partition coefficient f iodide between muscle and plasma in shock (10 d gs) Through a short midline incision the renal pedicles (rtery vein and urether) were ligated. Where iodide was used as a tracer the thyroid was blocked.

50μ Ci N ^{131}I[10] or 250μ Ci ^{51}Cr EDTA[10] (^{51}Chromium-ethylene-diaminetetraaceta e a chelate complex with Chromium (III) which does not penetrat muscle cells or erythrocytes and with capillary permeability properties similar to those of sucrose (Trap-Jensen and Lassen 1970)) was injected intra enously and the dogs were followed by repeated duplicate blood and plasma samples and muscle biopsies d ring control period and during periods according to the experi mental protocols of I II III I some of the dogs the equilibration times between muscle and plasma were controlled by injection of N ^{131}I and repeated sampling.

The partition coefficients between tissue nd plasma were calculated as acti ity per g muscle/ activity per ml plasma. Acti ity of iodide in erythrocytes was calculated f om plasma activity whole blood activity and hematocrit.

Xenon clearance and venous outflow in isolated gastrocnemius muscle (10 dog). Through medial incision along the thigh and the calf the femoral and popliteal vessels were mobilized and the vessel to the gastrocnemius muscle isolated. The gastrocnemius was isolated by blunt

10) AB Atomenergi, Studsvik, Sweden.

dissection from neighbouring structures and the achilles tendon and the proximal attachments to the tibia with their sesame bones cut All visible fat was removed. The muscle was wrapped in moist gauze and a polyethylene sheet and attached again at its original place in the calf. Temperature was controlled by a heating lamp. The femoral vein was cannulated by a T-tube, where flow could be diverted into weighed heparinized tubes. The flow was set at different levels by graded arterial compression.

With the flow set at different levels between 1 and 10 ml/min/100 g the xenon activities of a proximal (B) and a distal (A) depot (fig 5 p. 31) were followed simultaneously and the xenon clearances calculated as described. 4 dogs of 10 were given 150 ml 10% dextran 40 in an attempt to minimize the flow disturbance by the operative trauma (see Comments in Results)

Control experiments on PS (3 dogs). The dogs were anaesthetized, catheterized and followed by repeated clearance measurements with and without vasodilator papaverine during 8—10 hours.

Shock I Hemorrhag (17 dogs). After induction of anaesthesia, catheterisation and 2 hours of control observations the dogs were bled calculated amount 45—50% of the blood volume (assumed to be 8% of body weight (Wiggers 1950, Chien 1958)) within 30—60 minutes, followed 4—5 hours later by a second smaller bleeding 5 / *to accelerate the decompensatory phase of* shock. 1 supplementary dog (fig. 12, p. 36) was given 0.8 dextran 40/kg bwt as a fast infusion followed by a low infusion of the same agent 1 hour after the second bleeding.

Shock II Exteriorisation f the intestines (15 dogs) After catheterisation and control observations laparotomy and splenectomy were performed. The intestines were placed outside the abdomen under tension and wrapped in wet towels, soaked in warm saline. After shock period of 3 hours th intestines were replaced inside the abdominal cavity and the abdomen closed.

10 dogs (group A) were given dextran 40 1,5 g/kg bwt/15 min followed by 0,5 g/kg bwt/hour 5 dogs (group B) were only given slow saline in the post-shock period.

Shock III I f sion of dextran 1000 (5 dogs). After catheterisation and 2 hours of control observations dextran 1000 1,5 g/kg bwt in 15% solution was given within 5—10 min tes. After a shock period of 3 hours dextran 40 2 g/kg bwt was given w thin 20—30 minutes.

Shock IV Regional hypotension (9 dog). Through longitudinal incision the abdominal aorta and the iliac arteries were mobilized and isolated between the renal rteries and the inguinal ligaments. A clamp for graded occlusion of the aorta was placed just distal to the renal arteries. The A V pressure gradient to the hindlegs was measured with catheters in the aorta through the inferior mesenteric artery and in the inferior caval ein through small lumbal vein.

After preparation and control that maximal PS of iodide could be reached in the muscles of both the forelegs and the hindlegs, the abdominal aorta was compressed to a distal perfusion pressure 50 mm Hg. The occlusion was released after 3 hours. During the 3rd hoo of occlusion several manoeuvres such as riation of perfusion pressure (30—70 mm Hg), test of vasodilator and infusion of dextran 40 were performed in some of the dogs.

V Variation f perf sion pressure in vasodilated muscle (12 dogs). Group A (5 dogs) and group B (4 dogs) were prepared as described above (IV) I group C (3 dogs) the femoral artery and vein were mobilized and isolated between the inguinal ligament and the adductor hannel. Collateral circulation to the calf was occluded by screw clamp around the thigh; total occlusion was controlled by the cessation of flow and washout of isotopes with total arterial occlusion. Graded arterial and enous occlusion allowed perfusion of the calf at different distal perfusion pressures and venous pressures.

All dogs were given 150 ml 10% dextran 40 before preparation in an attempt to minimize the effect of the operati e trauma (which w th the nontreated animals (IV VI) led to the rejection of 5 dogs of 19 because of low maximal PS fter the preparation). After preparation several controls that maximal PS could be reached were performed. After injection of isotopes with vasodilation procedures and d ring the registration f the residual activities at the injection site the perfusion pressure was set t different levels for 3—5 minutes by arterial occlusion at normal

or increased enous pressure. An injection was accepted as successful only if the same clearance alue was reached at the end f the registration as t the starting point

Hematocrit was changed by bleeding the animals in small portions, substituting the blood with dextran 40 and the dog s own plasma

VI Variation of perfusion pressure in v sodilated muscle in shock Preparation was as described above (IV) Registrations were done as described under V Shock models I—III were studied. Analyses of material from (I) and (III) were supplemented by experiments on 3 dogs (model II) and 2 dogs (model I). Fo detail (VI) should be consulted.

Comments on results

The "iodide space" in skeletal muscle in shock[11])

The distribution of extracellular anions between muscle tissue and plasma was studied in shock models I—III using ^{51}Cr EDTA (Shock I), ^{131}Iodide and ^{125}Iodide as extracellular tracers Either ^{51}Cr EDTA or ^{131}Iodide was given 30—60 minutes before the first blood and tissue samples were taken. ^{125}Iodide was given by repeated injections and used as a test tracer for the rate of equi libration of iodide between muscle and plasma in shock.

Practically no ^{51}Cr activity was found in washed red cells The equilibrium ratio of ^{131}Iodide or ^{125}Iodide between plasma and red cells was variable the activity concentration within red cells was 0 4—0 8 that of plasma concentra

Table 2 The equilibrium distribution of ^{131}Iodide (and ^{51}Cr EDTA) between skeletal muscle and plasma in shock. $\frac{\text{Acti lty/g tissue}}{\text{activity/ml plasma}}$ in control and shock I—III. n = number of duplicat samples of muscle biopsies and plasma (S.D = standard deviation).

	Control period	Shock period 1st hour	2nd	3rd	4th	5th	Post shock period
Shock I							
^{51}Cr EDTA,	0.192	0.239	0.167	0.179	0.168	0.165	—
(3 dogs)	(S.D 0.013)	—	(0.031)	—	—	(0.019)	
	n = 8	2	10	2	2	8	
^{131}Iodide	0.198	0.229	0 181	0.176	0 164	—	—
(2 dogs)	(0.023)	(0.028)	(0.024)	(0.021)	(0.028)		
	15	8	8	12	24		
Shock II							
^{131}Iodide	0.244		0.223	0.201			0.194
(3 dogs)	(0.030)		(0.045)	(0.042)			(0.049)
	10		14	14			12
Shock III							
^{125}Iodide	0 186		0.185	0.236			0.206
	(0.018)		(0.024)	(0.027)			(0.043)
	6		4	8			8

11) in collaboration with Da id H. Lewis, Department of Surgery I Uni ersity of Göteborg.

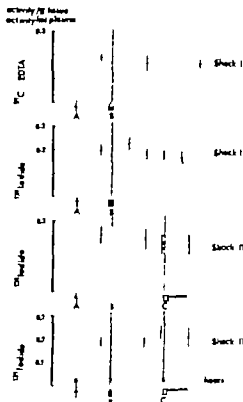


Figure 4 The partition coefficient between muscle tissue and plasma (activity/g muscle/activity/ml plasma) after equilibration f [131]Iodide or [51]Cr EDTA *in vivo*. The parti tion coefficients are given as mean values ± 1 S.D. from table 2, p. 21 Shock models I—III (see p. 5). Isotope administr tion t A. B, induction of shock by hemorrhage (I), exteriorization of the intestines (II) or in fusion of dextran 1.000 (III) Dextran 40 was given at C in shock II and III

tion. The equilibration to stable plasma — red cells quotients with [125]Iodide required 5—20 minutes *in vivo*. The experimental conditions were different from those of Tosteson (1959) who worked with beef and human red cells suspended in a chloride-phosphate medium at room temperature Tosteson found a higher equilibrium concentration within red cells than extracellularly and an initial *outflux* with a $t^1/_2$ longer than 10 seconds. In the present study the con centration was generally less in red cells than in plasma and the equilibration between plasma and red cells within a complex and probably slow mixing cham ber at body temperature much slower The omission of uptake of iodide in red cells during a single passage of blood through tissue in the calculated tissue-blood partition coefficient of iodide used in this study and the consequences of this assumption for the results have been discussed in papers I and V In control the muscle-plasma activity ratio was constant within less than 30 minutes. Repeated [125]Iodide injections gave stable tissue-plasma ratios identical to those of [131]Iodide within less than 45—60 minutes during the 3rd and 4th hour after hemorrhage.

During the first hour after bleeding (Shock I see Table 2 and Figure 4) tissue and plasma activities of [51]Cr EDTA and [131]Iodide fell approximately 30% a time lag in tissue activities. This indicated a dilution of plasma from areas

lower tracer concentrations and was probably the explanation for the higher tissue-plasma quotient found during the first hour after bleeding

The quotients of activity between muscle and plasma are given in Table 2 and Figure 4. The quotients were approximately 0.16—0.22 (cf. e.g. Wallace and Brodie 1937, Flear, Crampton and Matthews 1960, Agnew 1965, Crone and Garlick 1970, Gosselin *et al* 1971, Lassen and Sejrsen 1971) with no significant difference between ^{51}Cr EDTA and ^{131}Iodide in shock I. In neither shock model were the changes between control, shock (and post-shock) periods significant.

The partition coefficient muscle-plasma 0.16 used throughout this study is consistent with these values.

It is concluded that with the present results there was in neither shock model any indication of a redistribution of iodide within the tissue leading to an increased mean tissue concentration late in shock. Thus a systematic change of λ values of iodide in shock could not explain the differences in iodide clearances found in control, shock and after restitution to control conditions by dextran 40 infusion (I—III).

The small changes in "iodide space" in shock found here are consistent with the minor changes of 2—5% in sodium ion and sulphate spaces in skeletal muscle found in hemorrhagic shock (Newton, Pease and Butcher 1969, Johnson and Tucker 1968).

Xenon clearance and venous outflow in isolated gastrocnemius muscle in the flow range 1—10 ml/min/100 g[11])

Paradise, Swayze, Shin and Fox (1971) showed that surgical isolation of a skeletal muscle in the dog (extensor digitorum longus of the calf) led to a profound inhomogeneity of the perfusion even with hyperemia and suggested that this was due to oedema formation. The extent of this inhomogeneity is difficult to estimate in the present experiments on isolated gastrocnemius muscle. Maximal iodide clearance but not xenon clearance was reduced in the few double-isotope injections with vasodilator papaverine made on the group without dextran 40. The weight increase of the isolated muscle compared to the muscle on the intact side was 3—5%, same in both groups.

The evaluation of the activity time curves was done as described in detail in the chapter on methods. Two groups of experiments were done with the isolated gastrocnemius muscle preparation, one group (6 dogs) without any treatment except slow saline infusion during the course of preparation and measurements and one group (4 dogs) pretreated with dextran 40.

Comparison was made between simultaneous clearances at two injection sites, one distal A and one proximal B (fig. 5). The passage of activity from A through the detector field of B is not expected to interfere with the determination of xenon clearance at B as long as the washout rates A and B are approximately

11) In collaboration with David H. Lewis, Department of Surgery I, University of Göteborg



Figure 5 Comparison of simultaneously registered xenon clearances from a distal (A) and a proximal (B) local depot in the isolated gastrocnemius muscle. *Left panel* No dextran 40 before the preparation (open circles); *right panel*, dextran 40 given before the isolation of the muscle (black dots). The diagonal line is the identity line.

similar Xenon clearances Q_X and venous outflows Q were compared either in a Q_X vs Q diagram (fig. 6 lower panel) or in an E (extraction = Q_X/Q v flow (Q) diagram (fig 6, upper panel)

The comparison between two injection sites gave approximate consistenc between simultaneous clearances irrespective of time after injection or of flow changes during the registration The calculated regression lines were

Without dextran

$$Q_X\ (B) = 0.88 \times Q_X\ (A) + 0.79\ (n = 33\ \ r = 0.75)$$

Dextran 50

$$Q_X\ (B) = 0.64 \times Q_X\ (A) + 1.47\ (n = 47\ \ r = 0.64)$$

In both groups $r = 0$ ($p \leq 0.001$) However the scattering of values is considerable.

The comparison between xenon clearance and venous outflow gave very few xenon clearance values higher than flow (fig 6, lower panel) An approximate identity between xenon clearance and flow was seen at low flows

Without dextran:

$$E \neq 1\ (p \leq 0.001)\ (1 < Q < 2\ ml/min/100\ g)$$

Dextran 40

$$E \neq 1\ (p \leq 0.001)\ (1 < Q < 4\ ml/min/100\ g)$$

while at higher flows E was lower than 1 (fig 6, upper panel)
There was a considerable scattering of Q_X values in both groups (Fig. 6) The regression line were

Without dextran:

$$Q_X = 0.21 \times Q + 1.1\ (n = 66\ \ r = 0.74)$$

Dextran 40

$$Q_X = 0.48 \times Q + 0.86\ (n = 94\ \ r = 0.71)$$

In both groups $r = 0$ ($p \leq 0.001$)

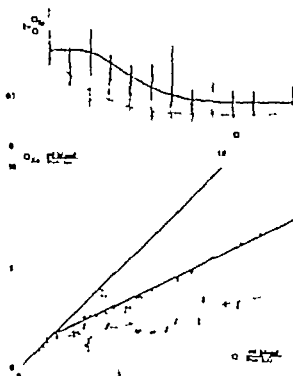


Fig e 6 Comparison of xenon cle r ance after local injection and enous outflow f isol ted g strocnemius muscle *Upper panel* Extraction (1 − xenon cle nce (Q_X)/venous outflow (Q)) vs Q Open circles without, black dots with dextran 40. Values are g en a grouped mean v lues ± 1 S D *Lower pan l.* Q_X vs Q The di gonal straight lines are from bove: The identity line the regres ion line for dogs gi en dextran 4 before preparation and the regres sion l ne (da hed) for dogs without dextr 4

The difference between the non-treated and the dextran 40 group was un expected and is difficult to associate with a countercurrent mechanism as the sole explanation for extractions lower than 1 in the flow range of resting skeletal muscle (Tönnesen and Sejrsen 1970) The possibility of increasing the extraction in the flow range 1—10 ml/min/100 g by pretreatment with dextran 40 before preparation is consistent with the hypothesis that uneven perfusion in resting skeletal muscle, perhaps accentuated by the surgical isolation procedure (Paradise *et al* 1971) contributes to an extraction lower than 1 for xenon from a local tissue depot.

It is impossible to draw any exact conclusions regarding the effect of uneven perfusion on Q_{X}/Q in shock from the present results but the failure to increase iodide clearance by papaverine in the isolated muscle as well as in shock (I III) points to similar transport disturbances in both situations.

Although the estimation of volume flow by xenon clearance is rather inexact, as shown here xenon clearance may still be used as an estimation of flow at flows below 3—4 ml/min/100 g and — while underestimating flow systemati cally at higher flows — is positively correlated to flow at flows between 4 and 10 ml/min/100 g The considerable scattering of xenon clearances has been inter preted as due to an uneven perfusion and a non-steady state washout from an

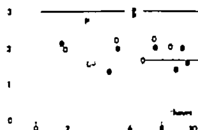


Fig. 7 Control experiments in dogs anaesthetized with Penthotal.* PS_P capillary diffusion capacity of iodide in ml plasma/min/100 g p indicates papaverine 0.8 mg/ml in the injectate, 0 no vasodilator The horizontal lines are mean values with and without papaverine of the control period of paper (I).

inhomogeneous system and the possible interference with the circulation by the injection. The approximate agreement between xenon clearance and volume flow found by Tönnesen and Sejrsen (1970) at flows above 15 ml/min/100 g has been accepted here.

Thus xenon clearance has been used here as an estimation of *vascular perfusion* — with due awareness of the underestimation of flow within the flow range 4—15 ml/min/100 g (Cf Discussion to paper I) Similar xenon clearances have been interpreted as indicating the same perfusion within the injection areas. *It is evident that an underestimation of flow by Q_{X}, will effect an overestimation of PS of iodide at high iodide extractions while being of no consequence at low extractions*

Control experiments[11])

During an observation time of 8—10 hours there were no consistent changes in hematocrit, PS of iodide and xenon clearance (*Cf* Wolpert *et al* 1970) The response to vasodilation by papaverine in the injectate did not change (fig 7)

Shock experiments[11])

Various aspects of shock model I—IV The mode of estimation of disappearance rates from curves with changing slopes is amply illustrated in figure 8—11 Figures 8 and 9 refer to the same dog as in Figure 1 paper (I) Comparison between Figures 8 and 1 (I) illustrates the analysis of the washout curves leading to step-formed curves of Q_X and PS of iodide and also clearly suggests that drastic changes in Q_X and PS occur which can be seen in spite of the variability of the experimental data.

11) I III in collaboration with David H. Lewis, Department of Surgery I University of Göteborg

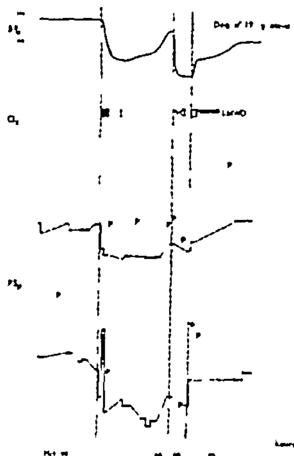


Figure 12. Mean arterial blood pressure mm Hg, xenon clearance Q_X ml blood/min 100 g, capillary diffusion capacity or permeability-surface area product of iodide PS_I ml plasma/min/100 g and hematocrit Hct %, in one dog subjected to hemorrhage at H I and H II. p indicates vasodilation by papaverine 0.8 mg ml in the injectate. LMWD dextran 40 0.8 g/kg bwt/15 minutes in 10% solution followed by a slow infusion of the same agent.

between iodide and xenon curves (Cf starred segments of curves early and late in shock in figures 8 and 10)

The decreasing response of iodide washout to local papaverine during progressive shock is illustrated in figs. 9 and 11. Figure 11 illustrates clearly the relative uniformity of xenon curves independent of the phase of shock or post-shock in a shock model with hemoconcentration but only slight hypotension as a typical feature. In a shock model with even more accentuated disturbances of the blood rheology the xenon slopes would be reduced as well (III). With the more accentuated hypotension (and a higher sympathetic activity opposing papaverine vasodilation. Cf Table I (I) and Table 4 p 41) of hemorrhage xenon *curves with papaverine had a more variable slope* (fig. 9).

In spite of the difficulties of the interpretation of the washout curves the proposed analysis (p 24) resulted in a strikingly similar time pattern of Q_X, and PS_I for all shock models (fig 3 (I) fig 1 (II) fig. 2 (III)[12]) and fig 3 (IV)) except in the early phase where shock model I and IV gave an instability of PS

12) Note that in paper III PS of iodide are given in ml blood min/100 g.

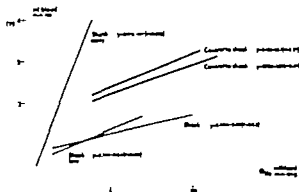


Figure 13 Iodide clearance (C_I) ml blood/min/100 g vs. xenon-clearance (Q_{Xe}) ml blood/min/100 g. Regression lines of control registrations to shock I and II of values with high iodide clearance early after bleeding in shock I (see text, p. 37 and (I)) and of registrations late in shock I and II. Note that the line of "shock I early" is close to the identity line.

of iodide not seen in shock models II and III. The time pattern is characterized by a reduction of Q_X and PS_I and a decreased (II) or wholly lost (II III IV) response of PS_I and a slightly (I IV) or clearly decreased (II III) response of Q_X to vasodilation by papaverine *(cf* Table 4)

The restitution to control conditions with dextran 40 is illustrated in figure 11 *(cf* II III IV VI). The reversal of PS_I reduction in hemorrhagic shock by dextran 40 is illustrated in figure 12 (which refers to one dog mentioned in (I)).

The variability of iodide and xenon clearances has been handled by plotting paired clearance values in the iodide-xenon clearance diagram (fig 4 5 (I) fig 2, 3 (II) fig. 3 (III), fig 2 (IV)) indicating the range within which experimental values were found by shaded areas. A non parametric rank test (such as Wilcoxon's test) applied to the comparison of iodide clearances in control and shock regardless of xenon-clearance (e. g II fig. 2, 3) would suggest a difference at a high significance level while the same comparison between xenon clearances regardless of iodide clearance would not indicate any difference. In addition the F-test for comparison of variances showed the variance of Q_X = the variance of C_I ($p \leq 0.001$) both in control and shock that is the variance of xenon clearance is larger than the variance of iodide clearance.

An alternative treatment would be the calculation of regression lines of C_I/Q_X (fig. 13) A selection of data with high iodide extraction (I IV observe that such a selection is arbitrary (k_I/k_X > 2, *Cf* IV)!) gave a slope close to one and a high correlation coefficient between C_I and Q_{Xe}. In contrast the correlation between C_I and Q_{Xe} was not significant at 1% level either in control or shock. This is an alternative way of illustrating the relatively unchanged C_I (Cf fig 4 (I) fig. 2 (II)) at different Q_X both control and shock. If Q_X can be accepted as coming closer to perfusion than C_I these results indicate *diffusion limited transport of iodide in resting skeletal muscle in control with further reduction of iodide diffusion in shock* Consideration of a possible underestimation of perfusion by Q_{Xe} (pp. 20 32, I V) would only strengthen these arguments further

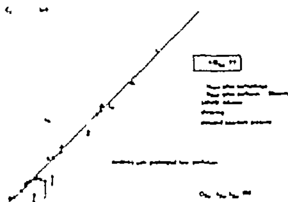


Fig e 14 Accumulated data w th h gh iodide extractions within the rest g flow range of sk letal muscle Iodide clearance (C_I) ml blood m / 100 g t xenon cle nce (Q_X) ml blood/min/100 g h, early fter he morrhage (I) o, early after gr ded aortic compression (IV) Ls d t from fig 12 and simil experiments during in f sion of dextran 40 after hemorr hage; Lo inf ion of dextran 40 at a perf sion pressure to the hindlegs 50 mm Hg (IV) c arrow elevated capil lary pressure (see text, p. 39 and tabl 3 p 38). The diagonal line t the identity line between C_I and Q_X.

If iodide transport is predominantly diffusion limited in resting skeletal muscle both in control and in shock the extraordinary situations with *identity between C_I and* Q_X become of special interest. Very low Q_X (less than 1 ml/min/100 g) were always connected with an iodide extraction close to 1 (see several figures in paper I—IV) In figure 14 data with approximate identity between C_I and Q_X have been gathered They include the unstable state early after hemorrhage (h) or graded occlusion (o) shivering (sh) infusion of dextran 40 (during the infusion) in hemorrhagic shock (L_s Cf fig 12) and at low

Tabl 3 Different f actional disappear nce rates of iod de t unchanged enon washout t low flows (preparation a group C (V)).

Time f straight line segment min.	P mm Hg	P_v	$P_a - P_v$	k_I min—	k_X	k_I/k_X	Q_X ml/min/ 100 g
5	75	12	63	0.013	0.015	0.88	1.1
10	120	60	60	0.029	0.015	1.96	
9	78	11	67	0.026	0.018	1.43	1.3
9	140	75	65	0.033	0.018	1.78	
12	75	12	63	0.0080	0.011	0.73	0.8
16	125	60	65	0.016	0.011	1.43	
5	104	19	85	0.043	0.075	0.57	5.3
5	118	42	76	0.075	0.075	0.99	
5	80	16	62	0.039	0.054	0.73	3.8
5	140	80	60	0.054	0.054	1.00	

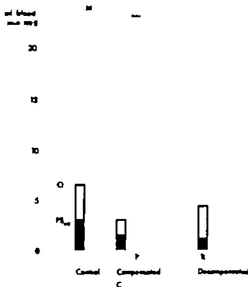
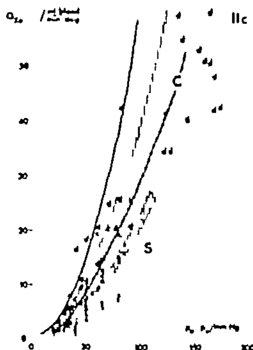


Figure 15 Shock I Comparison of xenon-clearance (Q_X) ml blood min/100 g and capillary diffusion capacity or permeability-surface area product of iodide (PS_{WB}) ml whole blood/min/100 g R resting skeletal muscle, no vasodilator P vasodilator papaverine in the injectate 0.8 mg/ml in the injectate. A, C, F control period and shock periods after hemorrhage according to (I).

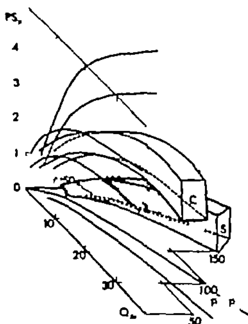
perfusion pressure (L_{∞}, Cf fig. 3 (IV)), and increased transmural pressure at unchanged perfusion pressure (Table 3 and Fig. 14 c arrow). The common factor of all these situations would seem to be increased capillary exchange area with undisturbed flow distribution and/or increased fluid transfer between tissue and capillary This suggests that the augmentation of tissue-blood transfer by net fluid exchange described by Lundgren and Mellander (1967) in hyperemic muscle might also be working at or below resting flow levels in skeletal muscle. The possibility of such an augmentation indicates that there is *no diffusion equilibrium between tissue and blood for substances such as iodide except at very low flow levels* or under conditions such as those of fig 14

The mean values of Q_X and PS_I in progressive hemorrhagic shock (I) with and without induced vasodilation by papaverine are summarized in figure 15 The decrease of PS out of proportion to the decrease of Q_X, is very clear As an example statistical comparison of "Control R" and "Decompensated P" (data, see Table I (I)) gave PS_I (Control R) = PS (Decompensated P) ($p \leq 0.001$) while there was no difference between mean values of Q_X.

The role of the perfusion pressure for tissue-blood exchange in the passive vascular bed (IV—VI) The complex interplay between overall perfusion pressure the small and changing pressure gradients within the capillary bed, the vascular geometry and the blood microrheology described in the introduction, suggests an instability of flow distribution in the vasodilated vascular bed at decreased perfusion pressure. The tissue-blood transport patterns of iodide and xenon have been analysed from this aspect in vasodilated skeletal muscle in control and shock (IV—VI) with PS of iodide representing diffusion (which may be



F g e 16 Shock II. Xenon (Qx) ml blood min/100 g in asodilated skeletal muscle (papa erine) at d fferent perfusion pressu res. c control period, shock period 1—2 *hours after exteriorisation of the intestines,* d post-shock period, 30—90 minutes after i fusion of dextra 4.. The curved lines indic t the range of Qx of the material of (V).



F gure 17 Shock II The relation between perfusion pressure $p_a - p_v$ mm Hg, xenon clearance Qx ml blood/min/100 g and capillary d ffusion capacity of lodide PS ml plasma/min/100 g in asodilated skeletal muscle (papa erine) in control (C) and shock II (S) Experimental alues were found within the olumes bounded by the curved square "tubes The projection of these volumes on to the ertical and hori sontal planes are given in figures 5 and 6 (V) figure 1 II A, II B (VI) and figure 16

closely related to flow distribution see Background and Discussion) and Q_x representing perfusion

The relation between perfusion pressure, perfusion (Q_x) and diffusion capacity (PS_l) under control conditions has been illustrated in diagrams of Q_x/perfusion pressure (fig 16) PS_l/Q_x (fig 5 (V)) and PS_l/perfusion pressure (fig 6 (V)) The three variables may be combined in a 3-dimensional diagram (fig 8 (V)) which gives a fairly detailed picture of the diffusion perfusion relations and the transport capacities of diffusion-limited and perfusion limited substances in the passive vascular bed of skeletal muscle at different perfusion pressures. Observe that during the experimental conditions of (V) these results were independent of hematocrit between 10 and 50%

The PS_l/Q_x/perfusion pressure relations in vasodilated skeletal muscle were changed in shock (figure 3 right panel (IV) figure 1 2 (VI) fig. 16) and is further illustrated in 3 dimensions in fig. 17 The diagram of figure 17 has been drawn by combination of fig. 8 (V), fig 1 II A and II B (VI) and fig 16

Table 4 Approximate magnitudes of perfusion and diffusion at different perfusion pressures in resting and vasodilated skeletal muscle in shock models I—IV Perfusion Q_x ml blood/min/100 g Diffusion PS_l ml plasma/min/100 g O = resting muscle no added vasodilator P = papaverine 0,8 mg/ml in the injectate. NP = no preparation except catheterization.

		Perfusion pressure mm Hg	Q_x ml blood/min/100 g		PS_l ml plasma/min/100 g		
			O	P	O	P	
Control		100	6	40	1.7—2.8	3—6	I—III (NP), V
		50 (acute red. 100→50)	—	15	—	3	V
Shock I	B	80	1.5	—	variable	—	I (NP)
	C	100	3	23[13])	0.9	1.9	
	F	50	4	10	0.7	0.6	
Shock II		100	3	30	0.8	1.5	II (NP)
		50	—	5	—	0.6	VI
Shock III		100	5	15	1.0—1.5	1.5	III (NP)
		50	—	3	—	0.7	VI and Appelgren, unpublished
Shock IV							
early		50	2—3	15	variable	3	IV V and Appelgren, unpublished
prolonged		50	3—5	10	0.6	0.6	

13) The possibility of residual vasotonus must be considered.

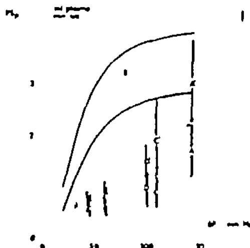


Figure 18 Shock I Accumulated values of capillary diffusion capacity of iodide PS_i ml plasma/min 100 g in vasodilated (papaverine, marked with lines) and resting (marked with dots) skeletal muscle in control (A) and in different shock periods according to (I). Values are given as mean values of PS_i ± 1 S D at the mean arterial blood pressure of each period (Table I (I))

(p 40) and refers to data from shock model II It should be fairly representative for shock models I III and IV as well (see fig 3 right panel (IV) fig 1 I A B III A B fig. 2 (VI)) with the following modifications (Table 4):

Shock model I IV: same reduction of PS_i but smaller reduction of Q_x than II at comparable perfusion pressures.

Shock model III same reduction of PS_i but larger reduction of Q_x than II at comparable perfusion pressures.

These types of diagrams may also be used to illustrate the progressive disturbances of transport in shock (fig. 18 shock I data from (I)) and the effect of different infusions (fig. 3 right panel (IV) fig 1 II A B fig 2 (VI))

The same diagrams may be used to illustrate changes in transport in the muscle in its resting state in shock as well (Fig 4 (I) Fig. 2 (II) Fig 3 (III) Fig 2 (IV) fig. 3 left panel (IV) fig 18 data from (I))

General discussion

The washout pattern from a local tissue depot of two radioactive tracers — one a lipophilic inert gas ^{133}Xenon the other a hydrophilic extracellular anion ^{131}Iodide — has been studied in four "shock" models I—IV for disturbed perfusion in skeletal muscle. In two groups of experimental models (described below by their main characteristics) disturbance of blood flow was induced by:

Increased vasoconstriction and/or low perfusion pressure

Hemorrhage (I)

Regional hypotension (IV)

Altered rheology of blood

Hemoconcentration induced by intestinal stasis (II)

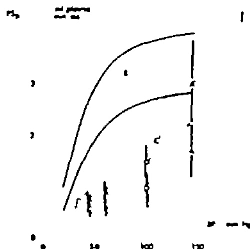
Red cell aggregation and increased viscosity induced by dextran 1000 (III)

Furthermore the washout pattern has been studied in detail in models I—IV as a function of perfusion pressure in vasodilated skeletal muscle (IV V VI).

The tissue-blood exchange of the two isotopes has been expressed as tissue clearances calculated from the fractional disappearance rates from the muscle (formula (3) p. 19) with the use of partition coefficients either from the literature (^{133}Xe) or calculated from the volume of the interstitial space (^{131}I see I V) The calculated values of the partition coefficient of iodide have been verified by an experimental study of the distribution of iodide between muscle and plasma in control and shock I—III

Iodide clearance was almost independent of xenon clearance except at the very lowest flows or in some special situations at fairly low flow (Fig. 14 pp 38) In each shock model there was with the progress of shock a reduction of iodide clearance at all xenon clearances except the lowest where the method could not discriminate between different iodide clearances. The relation between iodide clearance (C_I) and xenon clearances (Q_{Xe}) was interpreted as due to a diffusion limited transport of iodide with a reduction of the diffusion limit of iodide in shock. This reduction could also be expressed as a reduction of PS of iodide, the overall diffusion capacity or permeability-surface area product of tissue + capillary wall PS_I was calculated from C_I and Q_X using the simple tissue cylinder model (p. 17) for correction for interference of perfusion with diffusion. Perfusion was estimated by Q_{Xe}. The possible underestimation of perfusion by Q_X and the consequences of this have been considered (p 32—33)

There were more similarities than differences between the two groups of shock models. Late in shock PS_I was reduced both in resting and vasodilated skeletal muscle in all shock models. However shock models I and IV showed



F g e 18 Shock I Accumulated alues of capillary d ffusion c pac ty of iodide PSr ml pla ma min 1^0 g in vasodilated (p pa erine area marked w th lines) and resting (rea m k d w th dots) skeletal muscle in control (A) a d in different shock period a cord ng to (I). Values are gi en as mean alues of PSr ± 1 SD at the me n arte ial blood pres ure of each period (T ble I (I)).

(p 40) and refers to data from shock model II It should be fairly representa tive for shock models I III and IV as well (see fig. 3 right panel (IV) fig 1 I A B, III A B fig 2 (VI)) with the following modifications (Table 4)

Shock model I IV: same reduction of PS_I but smaller reduction of Q_X than II at comparable perfusion pressures.

Shock model III same reduction of PS_I but larger reduction of Q_X than II at comparable perfusion pressures.

These types of diagrams may also be used to illustrate the progressive distur bances of transport in shock (fig. 18: shock I data from (I)) and the effect of different infusions (fig. 3 right panel (IV) fig 1: II A B fig. 2 (VI))

The same diagrams may be used to illustrate changes in transport in the muscle in its resting state in shock as well (Fig 4 (I) Fig. 2 (II) Fig. 3 (III) Fig 2 (IV) fig 3 left panel (IV) fig 18 data from (I))

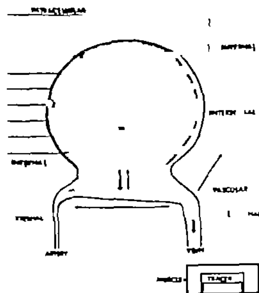


Figure 19 A schematic illustration of the relations between the transport system of skeletal muscle (interstitial compartment and vascular compartment) and the internal (intracellular) and external (outside the muscle) environment of the transport system. The relation between the volume of distribution of tracers after local injection and the whole muscle is indicated. Note that the vascular compartment should be interdispersed with the interstitial and the intracellular spaces.

High flows:

$$_{Ir} \approx k_I \approx \lambda_{Ir}$$

$$PS_I \approx PS^*_{Ir}$$

Low and intermediate flows

$$k_I \approx \lambda^*_{I+v} \approx Q \quad c_v/(b_v + b^*_v)$$

In these cases the amount of metabolite in the interstitial and the vascular compartments can be calculated from the steady state rate of formation of the metabolite and k_I. With increasing inhomogeneity of the interstitial compartment (and of cell metabolism) non-steady state conditions and/or small a v concentration differences for the metabolite these relations are no longer valid. However even if the fractional disappearance rate of the tracer can no longer describe exactly the complex transport situation of the metabolite it is still a measure of the tracer transport in skeletal muscle.

From k_I and k_X the parameters PS_I and Q_X were calculated in different experimental situations (pp 33—42, I—VI). Together with the perfusion pressure they gave a rather detailed picture of the dependence of spatial averages of tissue to blood diffusion of iodide and of perfusion on perfusion pressure in control and in shock (see e. g. fig. 17 table 4)

Further development of tracer kinetic methods at the macroscopic level

might perhaps decrease the scattering of transport parameters to some extent and define them more rigidly in relation to the time after labelling of the tissue but would hardly give a more detailed qualitative picture of the transport situation for small hydrophilic and lipophilic substances in skeletal muscle in control and shock than that given here Furthermore such improvements would not solve the problem of the relation between the transport kinetics of inert tracers and metabolites. This is a direct consequence of the heterogeneity of the transport system in skeletal muscle which must be accepted as a biological fact (see Background)

In principle a more detailed picture of the transport situation for metabolites would require new methods to handle the problems connected with the inhomogeneous system (*cf* Zierler 1961 1965) These problems may perhaps be attacked by calculations and *measurements* of concentrations at the level of small segments of the tissue-capillary cylinder combined with considerations of the distribution of the characteristic parameters of tissue cylinders (fig 20) Such methods of approach are only in their preliminary stage as yet (Knisely *et al* 1969 Bassingthwaighte *et al* 1970 Whalen 1971 Honig *et al* 1971)

B. The disturbed diffusional transport of iodide in shock — a distribution or a diffusion problem?

As shown in the present investigation the tissue-blood exchange is disturbed in shock both because of a *reduced perfusion* and a *reduced diffusion*

The reduction of *perfusion* in skeletal muscle in shock is well known and does not need any further comment. There is a rich literature on several aspects of perfusion in skeletal muscle in shock resistance, pre-/postcapillary resistance ratio fluid exchange and flow distribution (see e.g Mellander and Lewis 1963 Bond Manley and Green 1967 Renkin 1968 Stainsby 1968 Hollenberg and Nickerson 1970) but the literature is sparse on details of the *consequences* of disturbed flow distribution as such (Blalock and Bradburn 1930 Renkin 1964 1968 a, 1969 Nickerson 1970)

The reduction of *diffusion* in shock might be explained by a reduction of the available area for diffusion and an increase of diffusion distances because of *maldistribution of flow* Further possibilities for a reduction of PS of iodide would be an *increased diffusional hindrance within the interstitial space and the capillary wall* because of changes of the factor $\alpha \times D/\beta$ (i.e. physicochemical and microgeometrical factors of diffusion Cf p 13 and (I)) and *a disturbed extravascular perfusion*

While the literature on disturbances of flow distribution in shock is abundant, virtually nothing is known regarding the changes of the diffusional properties of the tissue or of the extravascular perfusion in shock These factors can therefore not be further elaborated in the discussion but they should not be forgotten in future investigations. However is must be emphasized that extra

vascular perfusion might be effective in the experiments with general or local vasodilation.

Therefore this discussion will deal mainly with disturbances of flow distribution as an explanation for the reduction of iodide transport in shock It is of interest that coarse calculations of the magnitudes of diffusion and perfusion (such as those summarized in table 1 p 14) suggest that the least marginals for reduction of transport are in *vascular perfusion* and *diffusion through the capillary wall*

For this reason the reduced diffusion in shock may be interpreted simply as due to a *reduction of the number of perfused capillaries* in shock.

PS_I may however also be reduced because of *uneven capillary perfusion* instead of a reduction of the number of perfused capillaries. The shape of the c/q_T curves of a tissue cylinder (formula (2) p 17) suggests that redistribution of flow between capillaries with the same ps/q_t will lead to a reduction of overall PS_I even at unchanged Q_t (Renkin 1964 1968 a, 1969 Ziegler and Goresky 1971)

Such a mechanism may also be the explanation for the decrease of PS_I at decreasing perfusion pressures and flows in vasodilated skeletal muscle (V VI) Such a decrease of PS might be expected to occur at *lower* perfusion pressures and flows for a substance of lower capillary permeability than iodide such as inulin, if there is a continuous transition from an even to an uneven flow distribution. This would occur at *unchanged* perfusion pressures and flows if the transition is due to a successively increasing number of capillaries with *total* cessation of flow (Alvarez and Yudilevich 1969 Crone and Garlick 1970) For the same reason the PS_I reduction in vasodilated skeletal muscle seen in shock above 60 mm Hg and 15 ml/min/100 g which was relatively independent of flow and perfusion pressure (VI) was interpreted as due to a reduction of the number of perfused capillaries in shock (*cf* the changes of CFC in vasodilated skeletal muscle in control, in hemorrhagic shock and after infusion of dextran 1000: Djojosugito *et al* 1970, Baeckström *et al* 1971)

A third possible mechanism for PS_I reduction is *large scale inhomogeneity of perfusion* that is inhomogeneity of perfusion between areas consisting of several adjacent capillaries separated by distances large enough to make interdiffusion negligible (*Cf* Background, p 11) This type of inhomogeneity may be partly accounted for by the random placement of injection sites and the method of curve evaluation (p 24)

Several rheological disturbances which could lead to either type of flow disturbance will be discussed below

The changes of tone of different vascular segments in a shock state would indicate the following transport changes:

a) a decrease of perfusion early in shock (I—IV) with a partial reve[illegible] control conditions, and

b) an increase of diffusion (after a very short reduction) because [illegible]

area for exchange (as registered by an increase of CFC)
(Lewis and Mellander 1963 Mellander and Lewis 1963 Lundgren *et al* 1964) The results of the present investigation are consistent with point a) but not with point b) if b) is not combined with the assumption of a disturbance of flow distribution through an open capillary bed in shock. Such considerations introduce blood microrheology into the discussion

When the experiments of Mellander and Lewis were repeated without priming the animal with dextran 40 (Baeckström *et al* 1971) the following changes were seen in hemorrhagic shock in the cat

a') a decrease of perfusion in the vasodilated muscle.
and

b') a decrease of CFC.
These changes were consistent with the directly observed changes of the microcirculation in cat skeletal muscle in hemorrhagic shock (Eriksson and Lisander 1972 b) They could be almost fully abolished by dextran 70 or by adrenalectomy before the bleeding but could also be induced by infusion of epinephrine in adrenalectomized cats in hemorrhagic shock (Baeckström *et al loc cit* Eriksson *et al loc cit*) The importance of the splenic blood brought into the circulation by autotransfusion in such a pattern cannot be excluded (Hardaway *et al* 1962 a, Ljungquist 1970)

Similar changes in flow and CFC as after bleeding were seen after the infusion of dextran 1000 in amounts much smaller than that given in III (Djojosugito *et al* 1970) here the combined effects of low perfusion pressure and disturbed blood rheology were seen illustrating the hysteresis phenomenon described in the introduction (p 00)

In all discussions on disturbances of flow distribution because of disturbed blood rheology it must be kept in mind that such disturbances are the result of a complex interaction between 1) changing *pressure differentials* within the vascular bed, 2) *vascular geometry* 3) *flow* itself and 4) *blood microrheology* (i.e. *plasma factors* such as fibrinogen (Chien *et al* 1971), fibrin monomers (?) other high molecular proteins such as macro-globulins, electronegative glycoproteins transformed by cleavage of electronegative groups (Rosato *et al* 1968) *and dextrans the size and deformability of blood corpuscles and their interaction* with one another and with the vascular endothelium and *hysteresis phenomena*) and late in shock 5) *intravascular coagulation*

Thus, besides low perfusion pressures there are several intravascular sources of disturbances of flow distribution most of them confirmed by vital microscopy also in skeletal muscle in shock, such as

cell wall adhesion of white cells
formation of platelet aggregates
increase of the inversion point for erythrocytes and platelet aggregates
reversible cell aggregation at low shear rates and venular stasis
and as late events:

formation of microthrombi
(e.g. Brånemark 1968 Strock and Majno 1969)

Most of these events are first seen in the small venules after bleeding and in shock (Knisely 1942, 1963 Gelin 1956 Eriksson and Lisander 1972 b) except an increased inversion point of erythrocytes which has hitherto not been defined *in vivo*

White cell adhesion to vessel walls seems to be an early event in hemorrhagic shock (Eriksson *et al* 1972 b *cf* Illig 1961) The possibility that other blood corpuscles such as platelets adhere to vessel walls is not settled experimentally

The formation of platelet aggregates in hemorrhagic shock in the dog (Ljungquist 1970) is triggered by plasma catecholamines in shock and closely related to the release of platelets from the spleen during bleeding The *in vivo* effect of low shear rates and tissue acidosis on the formation of platelet aggregates and the *importance of an increased inversion point for platelet aggregates* is not clear However platelet aggregates are certainly a part of the flow distribution disturbance seen by vital microscopy in cat skeletal muscle in hemorrhagic shock (Eriksson and Lisander 1972 b)

The increase of the inversion point has been described by *in vitro* experiments with narrow tubes (Weed and Lacelle 1969) or slits (Dintenfass 1971) and micropore filters (e.g. Chien *et al* 1971) The Fåhreus-Lindquist effect (1931 *Cf* Dintenfass 1971) is effective from vessel diameters 600 μ down to 10 μ and at hematocrits between 90 and 10 % However a further decrease of tube diameter will effect an increase of blood viscosity relative to plasma with a *sharp inversion point* at *2—5 μ* (Dintenfass 1971)

The inversion point is dislocated towards larger tube diameters for platelet aggregates as well as for erythrocytes at decreased pH or with storage in the refrigerator (e g Weed and Lacelle 1969 Dintenfass 1971 Goldstone *et al* 1971 *cf* Nahas *et al* 1960 Manger *et al* 1962) The inversion point for the red cell is closely related to its deformability (Weed and Lacelle 1969 Chien 1971 Dintenfass 1971) This mechanisms is the basis for *on-flow disturbances of flow distribution* together with plasma-skimming (Gelin 1956, 1963)

It is well known that reversible red cell aggregation occurs at low shear rates and is related to fibrinogen and other high molecular weight substances in plasma (e.g. Gelin 1956 Chien *et al* 1971)

According to Gelin (1963) the *off flow distribution* of blood in in vitro-experiments was different from the on-flow characteristics. Off flow stasis was provoked by low perfusion pressure, aggregation of cells, increased viscosity and decreased flow rate. This *disturbed off flow pattern* of blood was counteracted by hemodilution with dextran 40 This is of special interest in relation to the changes of PS_I and Q_T in shock models II and III

The stagnation of blood by the several mechanisms described above will lead to an unsharp and vaguely defined transformation to a graver flow disturbance with elements of platelet aggregation white cell adhesion, red cell agg

regation and fibrin deposition leading to *formation of microthrombi* (Knisely *et al* 1942 Knisely 1963 Hardaway 1966 Leandoer 1968 1969 Eriksson *et al* 1972 b)

Various combinations of these changes of blood microrheology will lead to a disturbed flow distribution which might be the explanation for the diffusional transport reduction seen in shock models I—IV of the present investigation.

In all shock models the transport disturbances could be reversed by dextran 40 infusion. The disturbances were not seen in dogs given dextran 40 before the bleeding (Appelgren, unpublished) This finding stresses the importance of disturbances of flow distribution for a disturbed tissue-blood exchange In this connection it is of interest to note the independence of PS_I to hematocrit (10—50 %) under control conditions in dogs primed with dextran 40 (V)

This reversibility by dextran 40 speaks against inspissated microthrombi as an explanation for the transport disturbances. It is of interest to note that the same diffusional disturbance of iodide transport has been seen in hemorrhagic shock in afibrinogenemic dogs, (Lewis, Myrvold and Olsson 1972) and in in shock model II in dogs given large doses of acetylsalicylic acid, cortisone or Pluronic F-68® (Lewis 1971)

It is concluded from the discussion above that the reduction of diffusional transport from tissue to blood of iodide seen in several shock models is most likely due to a disturbance of flow distribution even though other factors such as changed diffusional properties of the interstitial space might also be working. The mechanism behind such a distribution disturbance is not fully clear but several microrheological mechanisms seem to work simultaneously

As the clinical shock shows the same transport disturbances as the canine shock (Lewis and Appelgren 1972) the implication of this would be that prolonged low perfusion states should be avoided by an early instituted intense treatment aiming at a anormalized tissue perfusion. This is now accepted as standard in shock treatment. If for some reason a prolonged low perfusion state has been at hand an attempt to reverse the transport disturbance by dextran 40 may be done (See Gelin in Ingelman *et al* 1969) It is however not claimed here that this would be the only treatment possible. Early in shock several infusates might well give the same good result (Honegger 1967 however *cf* fig. 2 (VI) and Stallworth *et al* 1969)

Furthermore, one important lesson of experimental circulatory physiology learnt from the present investigation, is that a large preparation might give disturbances of the skeletal muscle circulation of the dog. Such disturbances are not readily seen in overall perfusion but only in *parameters sensitive to changes of the dynamic flow distribution* (*Cf* Paradise *et al* 1971) Such disturbances might be avoided by priming the animals with dextran 40 However the administration of dextran 40 might lead to an entirely different situation in experimental shock (*Cf* Mellander and Lewis 1963 Baeckström *et al* 1971)

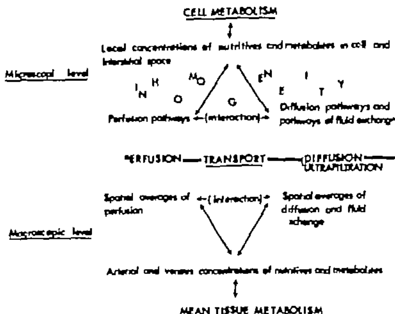


Figure 20 A schematic survey of the macroscopical and microscopical approach to transport disturbances between the cell and the organism in shock. The broad horizontal line symbolizes the principal borderline i the quantitative interpret tion of one set of observations (micro or macro) in terms of the other

C. The concept of nutritional flow

Accepting PS_I and Q_I as spatial averages of diffusion and perfusion it might be tempting to interpret $PS_I \times 100/Q_I$ or $C_I \times 100/Q_I$ (Cf fig 15 p 39) as an expression for % *nutritional flow* within the tissue in the sense that several authors have used extraction of $^{86}Rb^+$ (Cf Friedman 1966 1971) This would introduce unnecessary obscurity into the concept of nutritional flow The following description is suggested. *Nutritional flow is flow of blood through exchange vessels in relation to the material transport by perfusion and diffu sion* The *level* of nutritional flow is given by the perfusion value. The *quality* of nutritional flow would then be described by the compensation for the diffu sional hindrance by an efficient distribution of flow

D. Other approaches to a disturbed tissue-blood exchange in skeletal muscle in shock.

Shock at the level of the tissue has been described here as a grave disturbance of the mutual balance between the cell and the organism by transport distur bances or by cell damage (Introduction, p. 5) Such a disturbed balance may be approached by several methods besides the tracer kinetic method of the present investigation. The schema of Figure 20 suggests some possible approaches.

At the *macroscopic level* mean tissue metabolism has been studied by measuring a v-concentration differences of nutritives or metabolites and flow under *steady state* conditions (Cf Appendix I and Zierler 1961) e.g. oxygen consumption (e.g. Blalock and Bradburn 1930 Halmagyi *et al* 1969 Hermreck and Thal 1969 Wright *et al* 1971) or production of lactate in skeletal muscle in shock (e.g Greene and Willenkin 1963 Willenkin and Greene 1963) Spatial averages of perfusion and of diffusion of tracers may be measured as described in the present investigation

At the *microscopic level* ultrastructural changes of the skeletal muscle cell (e.g Holden *et al* 1963) as well as ultrastructural changes of the pathways for diffusion fluid exchange and perfusion in shock (e.g. Hammersen 1965 Strock and Majno 1969) have been described. Several aspects of altered cellular function and metabolism in shock have been studied metabolic energy stores (e.g Staples *et al* 1969 Lefer *et al* 1969) blocks of metabolic pathways (Schumer 1968) cell membrane transport of potassium ion and electrolyte distribution between cell and interstitium (Hagberg *et al* 1967 Haljamäe 1969 1970, Cunningham *et al* 1971) transmembrane potential (Creese *et al* 1958 Shires and Carrico 1966 Campion *et al* 1969 Cunningham *et al* 1971) and "intracellular" pH (Bell and Schloerb 1966 Brown Kim and Moorhead 1967)

Local concentrations such as electrolyte concentrations of exudates from the interstitium (Hagberg *et al* 1968 Haljamae loc cit, Cunningham loc cit) tissue pH (Lemieux Smith and Couch 1969 1969 a Wolpert Shaughnessy and Baccari 1970 Cf Smith *et al* 1969) and tissue oxygen tension (e.g. Groth 1966 Greene *et al* loc cit) have been measured and related to the same parameters of arterial blood and sometimes also of mixed or peripheral venous blood. Observe that these experimental parameters are usually averaged over an unknown tissue volume

Direct microscopic observations of the movements of diffusible coloured tracers of different molecular weight (Rous, Gilding and Smith 1930 Smith and Rous 1931 Hyman 1965 Arfors and Hint 1971) might give some insight the "dynamic morphology of transport by diffusion and perfusion within the tissue and of the diffusional pathways. The flow patterns in the microcirculation in skeletal muscle in shock have been studied in detail (see Background, p 11—12 and Discussion, pp 48—49)

The analysis of experimental results at the macroscopic level in terms of microscopic parameters *of the tissue or the synthesis of experimental results* at the microscopic level to averages for the whole tissue both stumble upon the problem already met in the analysis of the tracer kinetic data the lack of efficient quantitative descriptive methods for a complex biological transport system This limits the comparisons of experimental results at the microscopic and macroscopic level in fig. 20 this dividing line has been indicated by a broad horisontal line. The place of the local tissue clearance method is somewhere on this border line

Experimental observations on the consequences of a disturbed transport in shock have emphasized decreased tissue and vein oxygen tension and decreased tissue oxygen consumption, decreased tissue pH disturbed cellular metabolism disturbed electrolyte distribution and concentration gradients of potassium and hydrogen ion between interstitial fluid and arterial plasma. These observations can all be simply explained *by the low flow as such* Thus the "hidden tissue acidosis" in the sense of gradients of pH and metabolites between tissue and *arterial blood* can be explained solely by low perfusion. Changes in flow distribution and diffusion need not be introduced at this point.

However two points should be discussed in this connection

a) Concentration gradients between the tissue and peripheral regional venous blood

b) Dissociation in shock between oxygen consumption or lactate production in skeletal muscle on one hand and perfusion on the other

which cannot be explained by low perfusion alone. The importance of a steady state of tissue metabolism, flow and tissue-blood exchange at the time of sampling should be stressed again (Cf Zierler 1961)

Concerning point a) Most investigations of tissue-blood gradients in shock deal with comparison of tissue and arterial blood concentrations. Investigations in which comparison between tissue and peripheral venous blood in shock were done (e.g. Lemieux *et al* 1969 b) or in which differences between arterial and peripheral venous blood in shock were considered (Halmagyi *et al* 1970) indicated a smaller gradient between tissue and venous blood than between tissue and arterial blood. Still there seems to be a gradient between tissue and venous blood of a factor 3 in hydrogen ion in hemorrhagic shock (pH difference 0.5 Lemieux *et al* 1969 b) Potassium ion concentrations in interstitial fluid and arterial plasma differed by a factor 1.7 up to 3 in grave hemorrhagic shock in dogs (Hagberg *et al* 1968) Such results are consistent with the results of the present investigation where a reduction of PS_1 to 50 % in shock would double the tissue-blood gradient of substances with transport characteristics similar to iodide at diffusion limited transport and *unchanged tissue metabolism.*

Concerning point b) Oxygen consumption may be limited by cell metabolism, or by the oxygen available by perfusion and diffusion. In small rodents and in the cat O_2-consumption is transport-limited in resting skeletal muscle as indicated by the oxygen distribution within the tissue and by the perfusion dependence of oxygen consumption in the resting flow range (Whalen 1971 Honig *et al* 1971) The oxygen consumption in resting skeletal muscle of man and dog is limited by the cell metabolism, which means relatively high tissue oxygen tensions and independence of oxygen consumption to flow down to 2—3 ml/min/100 g (Landis and Pappenheimer 1963 Stainsby and Otis 1964 Wright and Sonnenschein 1965 Honig *et al loc cit*) Oxygen consumption in skeletal muscle in shock (cf Kniseley *et al* 1969) is difficult to analyse as it may

be decreased because of transport disturbances and decreased or increased because of a disturbed metabolism of the damaged cell. Some recent observations on oxygen consumption of the hindlimb of the dog illustrate the difficulties in deciding between the alternatives, disturbed cell metabolism or disturbed diffusional transport.

In different shock models (hemorrhagic shock after retransfusion (Halmagyi *et al.* 1969)) and septic shock (Hermreck and Thal 1969, Wright *et al.* 1971)) an increase of flow of 100—200 % was not followed by an increase of oxygen consumption.

The same difficulties are met with in the interpretation of lactate production and blood lactate levels (Cf. Alpert 1969/70). Hyperlactatemia is the rule in low perfusion states and is a complex resultant of increased production, decreased consumption and distribution problems. Washout phenomena with a temporary peak of lactate in plasma of arterial blood after dextran 40 infusion in shocked dogs (Litwin *et al.* 1965, Schumer 1967, Bergentz *et al.* 1969. Cf. fig. 1 (III)) suggested large areas of high lactate concentrations non-equilibrated with central blood in shock. Lactate levels in plasma in hemorrhagic shock continued to increase in spite of a normalisation of iodide clearance in skeletal muscle by an α-blocking agent but decreased at unchanged low iodide clearance when a β blocking agent was given (Makin 1971. Cf. Irving *et al.* 1968, Halmagyi *et al.* 1970). The production of excess lactate in the hindlimb of the dogs was unchanged at increased peripheral resistance by α adrenergic vasoconstrictors but increased at decreased peripheral resistance by hemorrhage + an α-blocking agent phenoxybenzamine (Ankeney *et al.* 1967) or hemorrhage + a ganglion blocking agent hexamethonium (Danoff and Greene 1964).

The conclusions from this last chapter must be that disturbances in single metabolic parameters in shock might be given several interpretations because of the multifactorial balance of tissue metabolism and tissue-blood exchange. The difficulties met in relating concentrations in tissue and blood and overall tissue metabolism in an unambiguous way to either transport disturbances or disturbances of cell metabolism have been commented upon.

Direct measurements of exchange rates between tissue and blood by tracer techniques and with considerations of overall diffusion capacities in relation to perfusion might help to elucidate these controversies. However, several problems concerning the relation between tracer turnover and metabolite turnover and the relation between observations at the macroscopic and at microscopic level remain to be solved. Therefore a combined approach at the macroscopic and at the microscopic level (fig. 20, p. 51) would be expected to give more conclusive results.

Summary and conclusions

The washout pattern from a local tissue depot of two radioactive isotopes — one a hydrophilic extracellular anion ^{131}Iodide, the other a lipophilic inert gas ^{133}Xenon — has been studied under physiological conditions and in states of disturbed perfusion in resting and vasodilated skeletal muscle in anaesthetized dogs.

The tissue clearances of iodide (C_I) and of xenon (Q_X) were calculated from the fractional disappearance rates of the two isotopes from the depot, measured by external gamma-counting. The relations between C_I and Q_X were studied in control and in four experimental shock models (I Hemorrhage, II Hemoconcentration by intestinal stasis, III High viscous state induced by dextran 1 000 and IV Regional hypotension). These relations were consistent with diffusion-limited transport of iodide both in resting and in vasodilated skeletal muscle in control and shock. Further analysis gave two transport parameters, one for *perfusion* (Q_X) the other for *diffusion* (PS_I). PS_I is the diffusional limit for iodide and is called the capillary diffusion capacity or the permeability-surface area product of iodide.

PS_I was reduced at comparable Q_X in all shock models. This reduction of PS_I could be reversed by infusion of dextran 40.

The diffusion and the perfusion parameters PS_I and Q_X were considered in relation to known properties of the interstitial space and the vascular bed in skeletal muscle during normal conditions and in shock. By estimation of the magnitudes of different elements of transport in skeletal muscle (i.e. vascular and extravascular perfusion, diffusion through the capillary wall and longitudinally and transversely to the capillaries through the tissue) it was found most likely that the reduction of overall diffusion of iodide (PS_I) seen in shock was due to a maldistribution of flow through the vascular bed. Some possible rheological mechanisms for such a flow disturbance were discussed.

These considerations lead to a description of nutritional flow in relation to tissue-blood exchange by diffusion and perfusion. The level of nutritional flow was given by the perfusion parameter Q_X while the quality of the nutritional flow was described by the way tissue perfusion overcomes the diffusional hindrance by an efficient distribution of flow (PS_I).

The relations between PS_I, Q_X, and perfusion pressure were studied in detail in vasodilated skeletal muscle in control and shock. PS_I was reduced about 50 % in all shock models at perfusion pressures above 60 mm Hg. There was an additional reduction of PS_I at perfusion pressures below 60 mm Hg both in control and shock. These reductions of PS_I were interpreted as due to

a reduction of the number of perfused capillaries at pressures above 60 mm Hg in shock An additional uneven capillary perfusion at perfusion pressures below 50—60 mm Hg might explain the additional reduction of PS_I seen both in control and shock Such disturbances would lead to some reduction of the overall perfusion as well To such a reduction of perfusion is added the reduction by an increased viscosity of blood in shock models II and III

An attempt has been made to analyse the transport of tracer by perfusion and diffusion in relation to the transport of metabolites of similar transport properties from tissue to blood. It was concluded that the turnover factor of a metabolite from the interstitial space under steady state conditions is in principle unobservable by tracer kinetic methods because of the different distribution of metabolite and tracer within the inhomogeneous transport system However the fractional disappearance rate of iodide may be used as an estimation of the turnover factor of water-soluble metabolites from the interstitial space under the limiting conditions of high flow if a homogeneous interstitial space can be assumed

Thus, the present investigation has emphasized the importance of the flow distribution within exchange vessels for the tissue-blood exchange in shock. In conditions of disturbed flow distribution as in progressive shock there is an impaired tissue-blood exchange which is out of proportion to the flow reduction. With such a disturbance an increase of flow will not increase the exchange of solutes unless the flow distribution is improved as well

Acknowledgements

This investigation has been supported by grants from the Swedish Medical Research Council (Contract No. Y-447 and grants B-69-2485-02 A and B-69-40-X 2042-03 A), from the United States Army through its European Research Office (Current Contract No. DAJA 37 72-C-0603), from the Faculty of Medecine, University of Göteborg and from Göteborgs Läkaresällskap. The generous support of Pharmacia AB, Uppsala, is also gratefully acknowledged.

It is a great pleasure for me to thank all those who inspired and supported this study:

Professor Björn Folkow Department of Physiology Faculty of Medicine, University of Göteborg; who first aroused my interest in the physiology of peripheral circulation.

Professor Lars-Erik Gelin, Surgical Clinic I and the Rheological Laboratory Faculty of Medicine, University of Göteborg; whose contributions within this field are now classical. His broad and long experience ranging from basic studies of the microcirculation to clinical shock studies gave the necessary background for this work. He followed my work throughout with untiring interest and friendly and constructive criticism.

Dr David H. Lewis, Surgical Clinic I Faculty of Medicine University of Göteborg; my teacher and co-worker during the progress of this work, who always had new suggestions and some kindly words to go with them in hours of doubt and standstill.

All my fellow-workers of the Rheological laboratory who helped in various ways to complete this work and who shaped the friendly atmosphere of our laboratory

Professor Per-Ingvar Brånemark, dr Elof Eriksson and dr Rhagnar Myrhage, Department of Anatomy University of Göteborg, for valuable help with advice on the functional morphology of skeletal muscle circulation.

Appendix 1

Tracer and metabolite turnover in the interstitial space and the vascular space of sk letal muscle.

The system studied (the transport system in skeletal muscle as described i detail in the introduction) is an open system w th n the field of the detector consisting of two *nonhomogenous* compartments, the interstitial compartment () and the ascular compartment ( ) in close contact. The en ironment of the system i di ided into two parts, one internal or intra cellular part (c) within the muscle and one external part (e) outside the muscle and outside the field of the detector (fig. 19 p. 45).

All symbols refer to 100 g muscle. V_i is th interstitial volume and V the vascular olume (plasma or blood volume); Q is the venou outflow $V_i \gg V$ The amount of substanc within compartments and are ndicated by b b (metabolite mother substance according to *Bergner*) and b_i, b_v (inert radioact e tracer). The unidi ectional flows of metabolite and tracer from a compartment 1 to compartment 2 are indicated by $\vec{\ }_{12}$ and $_{12}$. The turnover factor for metabolite transport from 1 to 2 is λ_{12} the time-dependent turnover function for tracer tra sport from 1 to 2 is $\varphi(t)_{12}$.

$$\vec{\ }_{12} = \lambda_{12} \quad b_1 \quad \text{(metabol te, } \textit{steady} \text{ state)}$$

$$_{12} = \varphi(t)_{12} \times b_1 \quad \text{(tracer on-st ady stat)}$$

An *unknown* part of the interstitial and ascular compartments are labelled *inhomogeneously* by th tracers (Fig. 19) The ^{133}X labelling also includes the cell lar compartment. The following equations will describe *formally* the transport of metabol tes from the cell and of the tracer from the interstitial compartment to the outside of the muscle

Metabolite steady stat

$$\vec{\ }_{ci} = \text{constant} = \vec{\ }_{ve} - \vec{\ }_{ev}$$

$$db\ /dt = O = \vec{\ }_{ci} - \lambda_{iv} \times b_i + \lambda_{vi} \times b$$

$$db\ \ dt = O = \lambda_{iv} \times b_i - \lambda_{vi} \times b + \vec{\ }_{ev} - \vec{\ }_{ve}$$

$$\vec{\ }_{ev} = Q \quad \text{arterial concentration c}$$

$$\vec{\ }_{ve} = Q \times \text{venous concentration } c_v$$

Tracer non-st ady state (observe that for ^{133}X ( ) should be changed to (+c)

$$_{ei} = b_i(t=O)/\ 1\ t \quad (O < t < 1\ t) \quad \text{(labelling procedure)}$$

$$_{ei} = O\ (t > 1\ t)$$

$$db_i/dt = \varphi(t)_{vi} \times b_v - \varphi(t)_{iv} \times b_i$$

$$db_v/dt = \varphi(t)_{iv} \times b_i - \varphi(t)_{vi} \times b_v + _{ev} - \zeta_{ve}$$

References

tonicity on the distribution f wate and solut l
8—70.

Vascular patterns in tissues and gr ft w th t p
6. 311—320.

the lactate metabolism. *H lt ac M d Act* 1969

letrick Heart capillary perme bility t l pd l bl m l
202. 45—58

eed Partition coefficient between various ti sue a d bl d
l 1967 *19* 7?—78

d *A.S. Luttell*, E perimental evidenc that a m t m n
esult in anaerobic metabolism. *Ann Su g* 1967 *166* 365 385

ards Clinical me surement of muscle blood fl w and apillary
duct according to Renkin by a double Isotope meth l (In S e
72.

ss Capillary permeab'l ty-su face a a pro luct (PS) f Renki
med scand 1968 *184* 281—282.

ers Simultaneous T scal m a u ement of regional bl d flow
in human y double isotope techniques (X
cally injecte *Mic ovasc Res* 1970 *2* 172—181

udies of sing fluorescent dextr n *Mic vasc*

f ltration a d diffu ion in experi

ele de k Studies on the
cand 1964 *129* 345—351

mt xchange of l y ln g n
5

f vidence f pl gging
ls (1971), pp 16—22

ts of vaw constriction

pathways in skelet l

model f a capillary

This relation at low flows have been assumed to be valid for xenon within the whole flow range of skeletal muscle.

Intermediate flow levels:

Overall turnover factor of metabolite in steady state:

$$\zeta^{*}_{(I+\,)s} = Q \times (c_v - c^{*}_v)/(b_i + b^{*})$$

Overall turnover function of iodide:

$$k(t)_I = E(t)_I \times Q \times (1 - Hct) \times b_I/(\lambda_I \times (b_I + b_s))$$

$$\approx E(t)_I \quad Q \times (1 - Hct)\ \lambda_I$$

$$= C(t)_I \times (1 - Hct)/\lambda_I$$

$$= C(t)_I\ (\lambda_I \times 100) \quad \text{(Cf formula 1 3 p. 00)}$$

where E_I is the (time dependent) extraction. From E and $Q_{Xe}(t)$ $PS(t)_I$ is calculated according to formula (2), p. 17 Observe that this PS_I value is comparable to PS_I at high flows *only if this correction is valid* However formula 2 seems to describe the experimental relation between E_I and Q reasonably well (see e.g. fig 2 and 3 (II)).

References

Agnew W.F. Effects of plasma tonicity on the d stribution of w ter and sol es brain and muscle. *Exp Neurol* 1965 *13* 58—70.

Algire G.H and *R.M Merwin*, Vascul r p tterns in tissues and grafts h t er chambers in mice. *Angiol* 1955 *6*, 311—320.

Alpert N.R., Regulation of the lactate metabolism. *H lsinc Med A ta* 1969 5 335—353

Alvarez O.A and *D.L Yudilevich* Heart capillary permeability to l pid inso ubl m cules. *J Physiol. London*, 1969 *202* 45—58

Andersen A.M and *J Ladefoged* P rtition coefficient between arious tissues a d blood in vivo. *Scand J clin Lab Invest* 1967 *19* 72—78

Ankeney J.L., *L.H Coffin* and *A.S Littell* E perimental evidenc that asoconstriction in hemorrhagic shock does not result in anaerobic metabolism. *Ann. Surg* 1967 *166* 365—380.

Appelgren K.L. and *D.H Lewis*, Clinical measurement of muscle blood flow and capillary permeability-surface area product according to Renkin by a double isotope method (In Swedish). *Nord. Med* 1968 *80* 1572.

Appelgren, K.L. and *D.H Lewis*, Capillary permeability-surface area product (PS) of Renkin in human skeletal muscle. *Acta med scand.* 1968 *184* 281—282.

Appelgren, K.L. and *D H Lewis* Simultaneous clinical measurement of regional blood flow and effective capillary surface n human skeletal muscle by a double-isotope technique Observations on the effect f locally injected norepinephrine *Microvasc Res.* 1970, *2* 172—181

Arfors, K E and *H Hint* Studies of the microcirculation using fluorescent dextran. *Microvasc Res.* 1971 *3* 440.

Arturson G and *S Mellander* Acute changes in capillary filtration and diffusion in experimental burn injury *Acta physiol. scand* 1964 *62*, 457—463

Arturson, G and *L Thorén* Capillary permeability in hemorrhagic shock. Studies on the blood-lymph barrier with dextran as test substance. *Acta Chi Scand*, 1964 *129* 345—351

Aukland K S Akre nd *S Leraand* Arteriovenous counter-current exchange of hydrogen gas in skeletal muscle. *Scand J lin. Lab Invest.* 1967 Suppl. 93 72—75

Baeckström, P B Folkow A.G.B Kovách B Löfving and *B Öberg* Evidence of plugging f the microcirculation following acute haemorrhage. I *Ditzel* and *Lewi (1971)*, pp 16—22.

Baeckstrom P B, Folkow E Kendrick B Löfving and *B Oberg*, Effects of vasoconstriction on blood viscosity in vivo. *Acta physiol. scand* 1971 a. *81* 376—384

Barlow T.E., A.L. Haigh and *D.N Walder* E idence for tw ascula pathways in skeletal muscle. *Clin Sci.* 1961 *20* 367—385

Bassingthwaighte J.B T J Knopp and *J.B Hazelrig*, A concurrent flow model for capillary tissue exchanges. I *Crone and Lassen (1970)* pp. 60—80.

Bell D J and *P R Schloe b* Cellular response to endotoxin and hemorrhagic shock. *Surg* 1966 *60* 69—76.

Bergentz S E., A Carlsten L-E Gelin and *J Krebs* Hidden acidosis in experimental shock *Ann. Surg* 1969 *16* 227—232.

Bergner P E. E Trac r dynamics II The l miting properties of the tracer system *J Theoret Biol* 1961 *1* 359—381

Bergner P E E. The gnificance of certain tracer k netical methods, especially with respect to the tracer dynamic definition of metabolic turnover *Act adiologic* 1962, Suppl 210. 1—59

Berman H J and G.P *Fulton*, The microcirculation a related to shock. I *Shock and Hypotension Pathogenesis and Treatment* (Mills and Moyer Eds.) Grune & Stratton, New Y rk and London. 1965 pp 198—219

Bl ks RJ and *D.F Davey* Osmotic responses demonstrating the extracellular character of the sarcoplasmic reticulum. *J Physiol. London* 1969 *202* 171—188

Blalock A and *H B adburn* Distribution of blood in shock: ygen content of venous blood from different local ties in shock, produced by hemorrhage, by h stamine a d by trauma. *Arch S rg* 1930. *20* 26—38

Bond R.F E.S Manley and *H.D Green* Cutaneous and skeletal muscle ascular response to hemorrhage a d irreversible shock. *Am. J Physiol.* 1967 *212* 488—497

B Jnemark P I Rheological a pects on low flow states. In *Shepro and F lton (1968)*. pp. 161—180

Brookes N and *D. Mack y* Diffusion f labelled substances through isolated rat diaphragm. *Br J Pharma* 1971 *41* 367—377

Brown E.B W.G Kim and *F.A Moo head* Intracellular pH during metabolic acidosis of intracellular nd extracellula origin. *Proc Soc Exp Biol. Med* 1967 *126*. 595—599

B rton A Discussion. In *Circulatory and Resp atory M T ansport A Ciba Foundation Symposium*. (Wolstenholme and Knight, Eds.). Churchill. London 1969 p. 133

Campion, D.S L.J Lynch F C Rector N Carter and *G.T Shires*, Effect f hemorrhagic shock on transmembrane potential *Surg* 1969 *66* 1051—1059

Cardon S.Z S F Oestermayer and *E.H Bloch* Effect of oxygen on cyclic red blood cell flow in anaesthetized mammalian striated muscl as determined by microscopy *Microvasc Res* 1970 *2*. 67—76.

Chambers, R. and *B.W Zweifach* Topography and function of the mesenteric circulation. *Am. J Anat* 1944 *75* 173—205

Chien S Quant tative eval tion of the circulatory adjustment of splenectomized dogs to hemorrhage *Am J Physiol* 1958 *193* 605—614

Chien S Blood rheology and ts rel tion to flow resistance and transcapillary exchange, with special eference to shock. *Adv Microcirc* 1969 2 89—103

Chien S S Usami R J Dellenback and *C A Bryant* Comparative hemorheology hematological implications f pecies differences in blood viscosity *Biorheol.* 1971 *8* 35—57

Cobbold A B Folkow I Kjellmer and *S Mellander* Nervous and local chemical control of precapillary sphincters in skeletal muscle as measured by hanges in filtration coefficient. *Acta physiol. scand* 1963 *57* 180—192.

Creese R., N.W. Scholes and *W.J. Whalen* Resting potentials of diaphragm muscle after prolonged anoxia. *J Physiol. London.* 1958 *140* 301—317

Crone C., The permeability of capillaries in arious organ as determined by use of the m dicator diffusion method. *Acta physiol scand* 1963 *58* 292—305

Crone C Capillary permeability — techniques and problems. I *Crone and Lassen (1970)*, pp. 15—31

Crone C. and *D Garlick* The penetration of inulin, sucrose, mannitol and tritiated water from the interstitial space in muscle into the ascular system. *J Physiol. London* 1970 *210* 387—404

Crone C. and *N.A Lassen* (Eds.) *Capillary Permeability The T ansfer f Molecules and Ions between Capillary Blood and Tissue* Proc. Alfr d Benzo Symp. II Cop hag n. J 1969 Munksgaard, Copenhagen. 1970.

Cunningham Jr J.N T Shires and J *Wagner* Cellular transport d fect in hemorrhagic shock. *Surg* 1971 *70* 215—222.

Danoff D.S and *N.M Greene* Vasodilation and the metabolic response *S rg* 1964 *55* 820—824

Day T.D The permeability of interstitial connecti e tissue and the nature of the inter fibrillary substance. *J Physiol London* 1952. *117* 1—8

Dintenfass L., Blood Microrheology — Visc sity Fact rs in Blood Flow Ischaemia and Thrombosis. Butterworths. London. 1971

Ditzel, J and D.T Lewis (Ed), *6th European Conference on Microcirculation Aalborg 1970.* S. Karger Basel. 1971

Djojosugito, A.M B Folkow B Oberg and *S White* A comparison of blood iscosity mea sured in itro and in vascula bed. *A ta physiol scand* 1970. *78* 70—84

Eriksson, E. and *R. Myrhag* Microvascular dimensions and blood flow in skeletal muscl *Acta physiol. scand.* 1972 a. I press.

Eriksson, E. and *B Lisander* Low flow states in sk letal muscle in cat. *Acta physiol. scand.* 1972 b. I press.

Fåhraeus R., The movement of water in surviving tissue t differen temperatures. A contribution to the pathogenesis of shock. *Act soc med Upsala.* 1955 *61* 107—135

Fåhraeus R. and *T Lindqvi t* The viscosity of blood in narrow capillary tubes. *Am J Physiol* 1931 *96* 562—568

Fick A., Ueber Diffusion. *Ann. Physik.* 1855 *94* 59—86.

Flear C.T.G R.F C ampton and *D.M Matthews* A in vitro method for he determination of the in lin space in skeletal muscle with observations on the composition f human muscle. *Clin Sci* 1960. *19* 483—493

Folkow B R.R Sonnenschein and *D.L Wright*, Loci f neurogenic and metabolic effects on precapillary essels of skeletal muscle. *Acta physiol. scand* 1971 *81* 459—471

Friedman, J.J Microvascular flow distribution and rubidium extraction. *Fed Proc* 1965 *24* 1099—1103

Friedman, J.J [86]Rb extraction as an indicator of capillary flow *Circ Res* 1971 *28* Suppl. 1 15—20.

Fulton, R.L., Adsorption of sodium and water by collagen during hemorrhagic shock. *Ann surg* 1970. *172* 861—869

Gelin L. E Studies in anemia of injury *Acta Chi Scand* 1956 Suppl. *210* 1—130

Gelin, L. E A method for studies of aggregation of blood ells, erythrostasis and plasma skimm ng in branching capillary tubes. *Biorheol.* 1963 *1* 119—127

Gersh I and *H.R. Catchpole* The nature of ground substance of connect e ti sue *Perspect Biol. Med* 1960 *3* 282—319

Goldstone J., P.M H tchins H Schmid Schönbein C U schel, E Sonnenblick and *R. Well* Correlation of microvasc lar and rheological factors in hemorrhagic shock. In *Ditzel and Lewis (1970)*. pp 150—155

Goresky C.A A linear method for determining liver sinusoidal and extra ascular olumes. *Am J Physiol 1963 204* 6.4—640

Goresky C.A., W.H Ziegler and *G G Bach* Capillary exchange modelling. Barrier-limited nd flow limited distribution. *Circ Res* 1970 *27* 739—764

Gosseli R.E Local effects of catechol mines on radioiodide clearance in skeletal muscle. *Am J Physiol* 1967 *210* 885—892.

Gosselin, R.E., The tissu tracer injection method for assessing capillary permeability I *Crone and Lassen (1970)* pp 218—227

Gosselin R.E and *G.R Stibitz* R tes of sol te absorption from tissue depots: Theoretical considerations. *Pflug Arch* 1970 a. *318* 85—98

Gosselin R.E. and *L.F Audino* Muscle blood flow and functional capillary density evaluated by isotope clearance *Pflug Arch* 1971 *322* 197—216.

G t R.T D rect observation of sk letal muscle blood essel (rat cremaster). *J Physiol. London* 1964 *172* 123—137

Greene N.M and *R.L W ilenkin* Skeletal muscle oxygen tension and metabolism during hemorrhagic hypotension and subsequent sopressor administration. *Yale J Biol. Med* 1963 *35* 429—438

G oth C -G The effect f infused plasma expanders and blood on the tissue oxygen tension. With special reference to the sign ficance f the flow properties of the blood. An experimen tal study in the rabbit. *Acta Chir Scand* 1966. *132*. 223—242.

Grotte G Passage of dextran molecules across the blood-lymph barrier *Act Chir Scand 1956* Suppl. 211 1—84

Gruber U.F Blutersatz Springer Verlag, Berlin 1968

Hagberg, S H H ljamäe and *H Röckert* Shock reactions in skeletal muscle II 1 tracellula potassium of skeletal muscle before and after induced haemorrhagic shock *Acta Chi Scand* 1967 *133* 265—268

Hagberg S H Haljamäe and H Röckert Shock reactions in skeletal muscle III The electrolyte content of tissue fluid and blood plasma before and after induced hemorrhagic shock. *Ann. surg* 1968 *168* 243—248.

Haljamae E Electrolyte Changes in Single M scle Cells induced by Experiment l Haemorrhagic Shock Thesis from the Department of Histology U iversity of Göteborg, Sweden. 1969

Haljamäe H "Hidden cellula electrolyte responses to hemorrhagic shock and thei significance. *Rev surg* 1970, *27* 315—324

Halmagyi, D.F J A.H Goodman and *I.R. Neering*, H ndlimb blood flow and oxygen usage in hemorrhagic hock. *J Appl Physiol* 1969 27 508—513

Halmagyi D.F M Kennedy and *D Varg* Hidden hypercapnia in hemorrhagic hypotension. *Anesth* 1970. *33* 594—601

Hammersen, F Zum Feinba der Muskelkapillaren in abgeschnurten Extremitäten der Ratte *Verh. Anat. Ges (Jena).* 1965 *115* 367—375

Hammersen, F The pattern of the terminal ascular bed and the ultrastructure of capillaries in skeletal muscle. In *Lubbers Luft Thews and Witzleb* (1968). pp. 184—197

Hammersen, F The terminal ascula bed in skeletal muscle with special regard to the problem of shunts. In *Crone and Lassen (1970).* pp. 351—365

Hardaw y R.M Syndromes of Disseminated Int avascular Coagulatio With Special Re ference to Shock and Hemorrhage Thomas. Springfield, Illinois. 1966.

Hardaway R.M W.H Brune E.F Geever J W Burns and *H.P Mock* Studies on the role f intra ascular coagulation in irreversible shock. *An S rg* 1962. *155* 241—250.

Hardaway R.M R.E. Nelmes J W B rns H Mock nd *P.T Trenchak* Role of the canine spleen in irreversible hemorrhagic shock. *Ann. Surg* 1962 a. *156.* 197—203

Hermreck A.S and *A.P Thal* Effects on vasoact ve drugs on blood flow and oxygen utilisation in septic tissue *Surg For* 1969 *20* 17—18

Hill A.V and *L. MacPherson,* The effect of nitrate, iodide and bromide on the d ation of the ctive state in skeletal muscle. *Proc Roy Soc (B).* 1955 *143* 81—102.

Hills, B.A Diffusion versus blood perfusion in limiting the rate of uptake of inert non-pola gases by skeletal rabbit muscle. *Clin. Sci* 1967 *33* 67—87

Hint H The pharmacology of dextran and the physiological background for the clinical use of Rheomacrodex and Macrodex. *Acta Anaesth Belg* 1968 2. 119—138

Holden W.D R.G DePalma W.R. Drucker and *A M Kalen* Ultrastructural changes in hemorrhagic shock. Electron microscopic study of h er kidney and striated muscle cells in rats. *Ann. Surg* 1963 *162* 517—536.

Hollenberg, N.K and *M Nickerson,* Changes i pre- and postcapillary resistance in pathogenesis of hemorrhagic shock. *Am. J Physiol.* 1970. *219* 1483—1489

Honegger H Microscopic observations of capillaries during hypovolemic shock in the dog In *Modified Gelatins Plasma Subst tutes. Proc Symp Berne Oct 1967* S Karger Basel 1969 pp. 364—366.

Honig C.R J.L. Frierr and *N N lson* O_2 transport and VO_2 in resting muscle signifi cance for tissue-capillary exchange. *Am J Physiol.* 1971 *220* 357—363

Hyman, C R le of specific microcirculatory elements in solute exchange. *Fed Proc* 1965 *24* 1095—1098

Hyman C and *J Lenthal,* Analysis f clearance of intraarterially administered labels from skeletal muscle. *Am. J Physiol.* 1962 *203* 1173—1178

Illig, L., Die terminale Strombahn. Capillarbett und Mikrozirkulation. *Pathologie und Klinik in Einzeldarst llungen* Bd X Springer Berlin-Gottingen Heidelberg 1961

Ingelman, B A. Gronwall, L. E Gelin and *R. Eliasson,* Properties and applications of dextrans. *Acta Acad Reg Scien Upsaliensis.* 1969 *12* 1—90.

Irving, M.H D Varga D J Gilett and *D F J Halmagy* The effect of adrenergic blockade upon shock-induced lactic acidosis in sheep. *Brit. J Surg* 1968 *55* 780—785

Jansson I Peripheral and myometrial ci culation in pregnancy Stud es in normal, toxaemic and diabetic pregnancies with venou occlusion plethysmography and ¹³³Xenon tissue lea rance *Acta Obstetr Gynecol g Scand* 1969 *48 Suppl 8* 1—32

Johnson G and *F.C T cker* Muscle fl id and electrolytes in shock *Surg For* 1968. *19* 29—31

Johnson J.A. and *T.A W lson* A mod l for capillary exchange. *Am. J Physiol.* 1966. *211* 1261—1263

Johnson J.A A model for capillary exchange with applications. In *Cro and La en (1970)*. pp. 206—214

Johnson P.C Regul tion f flow in the microcirculatory ystem. In *Malinm Lum Callahan and W rren (1970)*, pp 49—58

J seph N.R., M.B Eng l and *H.R Catchpole* I teraction of ions and connecti tissue *Biochim. b phys. acta* 1952. *8* 575—587

Karnovsky M J The ultrastructural basis of transcapillary exchange *J Gen. Physiol.* 1968 *52* 64s—95s.

Karnovsky M J Morphology of capillaries with special reference to muscle capillaries. I *Crone and Lassen (1970)* pp. 341—350.

Kedem O. and *A K tchalsky* Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. *B ochim. B ophys Act* 1958 *27* 229—246.

Kety S.S The theory and pplications of the exch ge of inert gas at the lungs and tissues. *Pharmacol. Rev* 1951 *3* 1—41

Kety S.S Det rminants of tissue oxygen. *Fed Proc* 1957 *16* 666—670

Kjellmer I The potassium ion as asodil tor d ring muscular exercise. *Acta physiol. scand* 1965 *63* 460—468.

Knisely M H I tra ascula erythrocyte aggregation (blood sludge). I *Handbook f Physiology Sect 2* (Hamilton nd Dow Eds.) Vol. III Williams & Wilkins, Baltimore 1963. p 2249—2292.

Knisely M H and *E.H Bloch* Microscopic observations of intravascular agglutination of red cells and consequent sludg ng of the blood in human diseases. *Anat Rec* 1942. *82* 426.

Knisely M.H D R. Rene and *D.F Bruley* The development and use f equation for predicting the lim ts on the rates of ygen supply to the cells of living tissues and organs. A con ribution to the biophysics of he lth and disease *A giol* 1969 *20* Suppl. *11* 1—56.

Kovers I.H M Acemoglu R. Cotton and *W R. Dru ker* Changes produced by hemorrhagi shock in the transport cha acteristics of the nterstitial fluid. *Surg Forum* 1970. *21* 36—37

Krogh A., The rate of d ffusion f gases through animal tissues, with some rema ks on the coefficient of in usion. *J Physiol London*. 1918/19 *52* 392—408.

Krogh A The umbe and distribution f capillaries n muscles with calculations of the oxygen pressure head necessary for supplying the tissue. *J Physiol London* 1919 *52* 409—415

Krogh A The supply f o ygen to the tissues and the regulation of capillary circulation. *J Physiol. London.* 1919 a. *52* 457—474

Kunze K Normal and critical oxygen supply to the muscle. In *Lubbers Luft, Thews and W tzleb (1968)*. pp. 198—208

Kushmer M J and *R.J Podola* Ionic mobility in muscle cells. *Science* 1969 *166*. 1297—1291

Landis E.M Discussion. In *Crone and Lassen (1970)* p. 370.

Landis E.M and *J.R. Pappenheimer* Exchange of substan es through the capill ry wall In *Handbook of Physiol. Sect* 2 (*Hamilton and Dow Eds.*) Vol II Williams & Wilkins, Baltimore. 1963. pp 961—1034

Langley J N., The rigin and course f the aso-motor f bres of the fro s foot *J Physiol London*. 1911 *41* 32—499

Lassen, N.A., Muscle blood flow in normal man and in patients with intermittent claudi cation evaluated by simultaneous Xe133 and Na24 clearances. *J Clin. Invest* 1964 *43* 1805—1812.

Lassen, N.A Capillary diffusion capacity of sodium studied by the clearance of Na 24 and Xe- 133 from hyperemic skeletal muscle in man. *Scand J clin Lab Invest* 1967 *18* Suppl. 99 24—26.

Lassen, N.A On the theory of the local clearance method fo measurement of blood flow including discussion of its application to vario tissues. *Acta med Sc nd* 1967 a. Suppl *472* 136—145

Lassen, N.A. and *J Tr p-Jensen* Theoretical considerations on mea urement f capill ry diffusion capacity in skeletal muscle by the local clearance method. *Scand J clin Lab Invest* 1968 *21* 108—115

Lassen, N.A. and J *Trap-Jensen* The alidity of the indicator dilution method for mea uring the capillary diffusion capacity for 51Cr EDTA in hyperemic skelet l muscle *E rop J cli Invest*. 1970. *1* 118—123

Lassen N.A and *P Sejrsen* Monoexponential extrapolation of tracer clearance curves in kinetic analysis. *Circ Res*. 1971 *29* 76—87

Laurent T.C The structure and function of the ntercellular polysaccharides in onnectiv tissue I *Crone and Lassen (1970)*. pp. 261—277

Leandoer L., Fibrinogen after massive haemorrhage Studies on coagulation and fibrinolysi of blood and lymph in dogs. *Acta Chi Scand* 1968 Suppl. 390 1—29.

Leandoer L and *S -E. Bergentz* Microthrombi and -emboli in hemorrhagic shock Th effect of AMCA. *Europ Surg Res* 1969 *1* 187

Lefer A.M *J.C Dow* and *R.M Berne* Cardiac and skeletal muscle metabolic stores in hemorrhagic shock. *Am. J Physiol* 1969 216 483—486.

Lemieux M.D *R.N Smith* and *N.P Couch* Su face pH and redox p tenti l of keletal muscle in graded hemorrhage *Surg* 1969 *65* 457—461

Lemieux M.D *R.N Smith* and *N.P Couch* Electrometric surface pH of keletal muscle in hypovolemia. *Am. J Surg* 1969 a. 627—631

Lewis, D H Radioactive rubidium ptake by a dog's hindleg d ring hemorrhagic shock *Excerpt. Med. Int. Congr Ser* 1967 *152* 32.

Lewis, D.H Effect of various pharmacological treatment schedules on the capillary blood flow and capillary transport function in dog skeletal muscle in surgical shock. I *Symposium on Protease Inhibitors in Shock Therapy* Schattauer Verlag, Stuttgart 1971 I press.

Lewis, D.H and *K.L. Appelgren*, Perfusion and diffusion in low flow states *Shock Symposium Brook Lodge* June 1971

Lewis D.H and *S Mellander* Competitive effects of symp thetic control and tissue meta bolites on resistance and capacitance vessels and capillary filtration in sk letal muscle. *Acta physiol. scand* 1962. *56* 162—188

Lewis D.H H Myrvold and *P Ols on* Hemorrhagic shock in af brinogenemic dogs. *Brit J Surg* 1972. In press.

Levitt D G Theoretical model of capillary xchange incorporating interactions between capil laries. *Am. J Physiol.* 1971 *220* 250— 55

Litwin M.S S E. Bergentz A Carlsten, L.-E. Gelin C. V Rudenstam and *B Söderholm* Hidden acidosis following intra ascular red blood cell aggregation in dogs. Effect of high and low viscosity dextran. *Ann Surg* 1965 *161* 532—538

Lundgren O J Lundvall and *S Mellander* Range of sympathetic disch rge and reflex vascular adjustments i skeletal muscle d ring hemorrhagic hypotension. *Acta physiol. scand* 1964 *62* 380—390.

Lundgren, O and *S Mellander* Augmentation of ti sue-blood transfer of solutes by trans-capillary filtration and absorption. *Acta physiol. Scand* 1967 *70* 26—41

Lübbers D W U.C. Luft G Thews and E W i leb (Eds.) *O ygen T a sport in Blood and Tissue* G Thieme Verlag Stuttgart. 1968

Makin, G.S Tissue perfusion nd lactic acidosis in haemorrhagic shock n experimental study *Ann. Roy C ll. S rg* 1971 *48* 114—121

Malinin T I B.S Linn, A.B Callaha and *W.D Warren* (Eds.), *Microcirculation, P rf sion and Transplantation of Organs. Academic Press.* New York and London 1970.

Manger W.M G.G Nahas, D V Habif and *E.M Papper* Effect of pH control and in-creased O_2 del very on the course of hemorrhagic shock. *Ann. Surg* 1962. *156* 503—508

M theson N.A Factors in tissue perfu ion. The microcirculation in shock. *Postgr med J* 1969 *45* 530—533

McLennan, H The diffusion of potassium, sodium, sucrose and inulin in the extracellular spaces f mammalian tissues *Biochim Biophys. Acta* 1957 *24* 1—8

Mellander S I teraction of local and nervous factors in ascular control. *Angiol.* 1971 *8* 187—201

M llander S and *B Johansson,* Control of resistance, exchange and capacitance functions in the peripheral circulation. *Pharmacol. Rev* 1968 *20* 117—196

Mellander S and *D H Lewis* Effect of hemorrhagic shock on the reactivity of resistance and capacitance essels and on capillary filtration transfer in cat skeletal muscle. *Circ Res* 1963 *13* 105—108

Moore J C and *C H Baker* Red cell and albumin flow circuits during sk letal muscle reactive hyperemia. *Am. J Physiol.* 1971 *220* 1213—1219

Nahas, G.G A M tt lman and *W.M Manger* The effect f buffering ACD blood with THAM on the survival f dogs transfused after mass e hemorrhage. *Fed Proc* 1960. *19* 54

Newton W T H.D Pease and *H R. B i ber* Sodium and sulphat distributions in dogs fter hemorrhagic shock. *S rg For* 1969 *20* 1—2.

Nichol J F Girling, W Jerrard E.B Claxton nd *A Burton* Fundamental instability of the small blood vessels nd critical closing pressures in ascular beds. *Am. J Physiol.* 1951 *164* 330—344

Nickerson M Vascular adjustments during the development of shock. *Canad med ass. J* 1970. *103* 853—859

Nicoll P.A and *R. Frayser* Physiological considerations of the microcirculation as related to shock. *Progr Cardiovasc Dis* 1967 *9* 558—570.

Ogston, A.G and *T.F Sherman* Effects of hyaluronic acid upon d ffusion of solutes and flow of solvent. *J Physiol. London* 1961 *156*. 67—74

Page E Ion movement in heart muscle: tissue compartments and the experimental definition of driving forces. *Ann. N.Y Acad Sci.* 1965 *127* 34—48

Page E. and *R.S. Bernstein* Cat heart muscle in vitro V Diffusion through a sheet of right ventricle. *J Gen. Physiol* 1964 *47* 1129—1140

Pappenheimer J R., Passage of molecules through capillary w lls. *Physiol. Rev* 1953 *33* 387—423

Pappenheimer J.R., Osmotic reflection coefficients in capillary membranes. In C one nd *Lassen (1970)*. pp. 278—286.

Pappenheimer J.R., *E.M Renkin* and *L.M Borrero* Filtration, diffusion and molecular sieving through peripheral capillary membranes. A contribution to the pore theory of capillary permeability *Am. J Physiol.* 1951 *167* 13—46

P ppenheimer J.R. and *A. Soto-Rivera* Effects osmotic pressure of plasma protein and other quantities associated with the capillary circulation in the hindlimbs of cats nd dogs. *Am. J Physiol.* 1948 *152* 471—491

Paradise N.F *C.R. Sw ys* *D H Shi* nd I *Fox* Perfusion heterogeneity in skeletal muscle using tritiated water *Am. J Physiol.* 1971 *220* 1107—1115

Paterson C.A and *D.M Maurice* Diff sion f sodium in extracellular space of the crystalline lens. *Am. J Physiol.* 1971 *220* 256—263

Perl W Heat and matter distribution in body tissues and the determination f tissue blood flow by local clearance methods. *J Theoret Biol.* 1962. *2* 201—235

Perl, W An extension f the diffusion equation to include clearance by capillary blood flow *Ann. N.Y A ad Sci.* 1963 *108* 92—105

Pharmacia AB Blood Flow Improvement Clinical and E perimental Data on Rheomacrodex® Pharmacia AB, Uppsala. 1968

Rand R.P and *A Burton*, Mechanical properties of the red cell membrane I Membrane stiffness and intracellular pressure. *Biophys. J* 1964 *4* 115—135

Rapaport S.I *A Saul*, *C Hyman* and *M.E. Morton*, Tissue clearance as measure f nutritive blood flow and the effect of lumbar sympathetic block upon such measures in calf muscle. *Circ lation.* 1952. *5* 594—604

Reeve E.B and *A.C. Guyton* Summary and comment. I *Physical Bases of Circulatory T ansport. Regulation and Exchange (Reeve* and *G yton* Eds.) Saunders, London, 1967 pp. 371—374

Renkin E.M Capillary permeability to lipid-soluble molecules. *Am J Physiol.* 1952. *168* 538—545

Renkin, E.M Effects of blood flow on diffusion kinetics in isolated, perfused hindlegs of cats. A double circulation hypothesis. *Am. J Physiol.* 1955. *183* 125—136.

Renkin E.M Transport of potassium-42 from blood to tissue in isolated mammalian skeletal muscles. *Am J Physiol.* 1959 *197* 1205—1210.

Renkin E.M Transport of large molecules across capillary walls. *Physiologist.* 1964 7 13—28

Renkin E M Normal regulation f tissue circulation. In *Effects of Anaesthetics on the Ci culation.* (Price H.L. and Cohen, P J Eds.) Thomas. Springfield, Illinois. 1964 pp. 171—181

Renkin E.M Neurogenic factors in microcircul tory low flow st tes. In *Shepro and Fulton (1968).* pp 139—148

Renk E.M Transcapillary exchange in relation to capillary circulation. *J Gen Physiol.* 1968 a. 52 96s—108s.

Renkin E M Exchange of substances through capill ry walls. In *Circulat y and Re pi atory Mass T ansport A Ciba Foundation Symposium.* (Wolstenholme and Knight, Eds.) Church'll, London. 1969 pp. 50—66

Renki E.M The nutritional-shunt flow hypothesis in skeletal muscle circulation. C c *Res.* 1971 *28* Suppl. *I* 21—25

Renki E.M and *S Ros ll* The i fluence of sympathetic ad energic vasoconstrictor nerves on transport of diffusible solutes from blood to tissues in keletal muscle *Acta physiol scand* 1962 a. *51* 223—240.

Renkin, E.M and *S Rosell* Effects of different types of vasod lator mechanisms on ascular tonus and on transcapillary exchange of diffusibl material in skeletal muscle *Acta. physiol scand* 1962 b. *54* 241—251

Renkin, E.M a d *S Rosell* Independent sympathetic vasoconstrictor innervation f arterioles and precapillary sphincters. *Acta physiol scand* 1962 c. *54* 381—384

R sat F.E., L.D Miller and *M Hebel* A biochemical definition of blood iscosity Its *possible significance in the pathophysiology of shock. Ann S rg* 1968 *168* 61—66.

Rous P H.P Gildi g and *F Smith* The grad ent f ascular permeability *J e p Med* 1930. *51* 807—830

Sang en W C. and *C W Sheppard* A mathem tical deri ation of the exchange of labelled substance betw n a liquid flowing in vessel and an external compartment. *B ll. Math Biophys* 1953 *15* 387—394

Skinner N.S and *W J Powell* Action of o ygen and potass um on ascular resistance of dog skeletal muscle. *Am. J Physiol.* 1967 *212* 533—540.

S hmid Schönbein H Hemodynamic sign ficance of red cell fluidity In *D tzel and Lewi (1970).* pp 2—7

Schmidt G W A mathematical theory of capillary ex hange as function of tissue structure. *B ll Math Biophys* 1952. *14* 229—263

Schmidt G W The time course f c pillary exchange. *Bull. Math Biophys.* 1953 *15* 477—488

Schumer W Physicochemical and met bolic effects f low molecula weight dextran in oli gemic shock. *J T auma* 1967 7 40—46.

Schumer W Localization of the energy pathway block in shock. *S rg* 1968 *64* 55—59

Sejrsen P Convection nd diffusion of inert gases in cutaneous, subcutaneous and skeletal muscle tissue. I *Crone and Lassen (1970).* pp. 586—596.

Sejrsen P and *K.H T nnesen* Concentration prof les for gas d ffusion in skeletal muscle in vitro and in vivo. *Act physiol scand* 1968 *74* 22A.

Shires, T and C.J Carrico, Current status of the shock problem. I Cur ent P blem in Surgery Year Book Med Publ. Chicago. March 1966.

Shepro, D and G.P F lton (Eds). M crocirc Lato s related to Shock Academic Press. New York and London. 1968

Smaje L., B.W Zweifach and M I taglietti Micropressures nd capillary filtration coeff cients in single vessels of the cremaster muscle of the rat. Mic ov sc R s. 1970 2 96—110

Smith R.N M.D Lemieux and N.P Couch Effects of acidos s and alkalosis on rf ce skeletal muscle hydrogen ion acti ty Surg 1969 533—538

Smith F and P Rous The gradient of ascula permeability III The condition in frog and chicken muscle, and in the mammalian diaphragm. J p Med 1931 53 195—217

Spalteholtz W Die Verteilung der Blutgefässe im M skel. Abhandl. Sächs Ges W ss., Phy Math. 1888. Kl. 14 509—528

Stainsby W N Autoregulation skeletal muscle during shock. In Shepro nd F lton (1968) pp. 41—49

Stainsby W.E. and A.B Oti Blood flow o ygen tension oxygen uptak nd o ygen transport in skeletal muscle Am J Physiol 1964 206 858—866.

Stallworth J.M., A Ramire B.A. Barrington and R.R. B adham, Hypovolemic shock Mic rocirculatory changes during and f er specific therapy Ann Surg 1969 169 694—703

Staples D C. T purla and E Blo A comparison of denosine triphosphate lev ls in hemorrhagic and endotoxic shock in the rat. S rg 1969 66 883—885

Starling, E.H On the absorption of fluid from the connecti e tissue spaces. J Physiol London. 1896. 19 312—326.

Streck P.E. nd G Majno. Microvascular changes i acutely ischemic rat muscle Surg Gyn. Obst 1969 129 1213—1224

Thompson, A.M H.M Cavert N Lifson and R.L. Evans, Regional tissue ptak f D_2O in perfused organs rat live dog heart nd gast ocnemius. Am. J Physiol. 1959 197 897—902.

Tønnesen K.H Blood Flow i the Skeletal M scle d ring Work measured by 133Xenon Clearance from Local Depot (in Danish). Thesis from the Faculty f Medicine U iversity of Copenhagen, Denmark. 1969

Tønnesen K.H and P Sejrsen, Wash-out of 133Xenon fter intramuscula jection and directly measured blood flow in skeletal muscle Scand J clin. Lab Inve t 1970. 25 71—81

Tosteson, D.C Halide transport in red blood cells. Acta physiol. scand. 1959 46 19—41

T p-Jensen J and N.A Lassen, Capillary permeabil ty for smaller hydrophilic tracers in exercising skeletal muscle in normal man and in patients with long-term diabetes mellitus I C one and Lassen (1970) p. 135—152.

Trap-Jensen, J O Korsgaard and N.A. Lassen Capillary permeabil ty in huma skeletal muscl measured by local injection of 51Cr EDTA and 133Xe. Scand. J clin Lab Invest 1970 a. 25 93—99

Ussing, H H Transport through biological membranes. Ann. Rev. Physiol. 1953 15 1—20

Walder D.N Vascular pathw ys in skeletal muscle. In Circulation in Skeletal Muscle (Hudlicka Ed) Pergamon Press, Oxford. 1968. p. 101—106.

Wallace G.B and *B.B Brodie* The distribution of administered iodide and thiocyanate in comparison with hloride and their relation to body fluids. *J ph rmacol exp ther p* 1937 *61* 397—411

Weed RJ and *P.L. Lacelle* ATP dependence of erythrocyte membrane deformability Relation to "in i surv al and blood storage In *Red Cell Membrane Structure and Function* (*Jamieson* and *Greenw lt* Eds.) J B. Lippincott. New York. 1969

Wells, R.E., Rheology of blood in the microvasculature. New Engl. J Med 1964 270 832—839 889—893

Whalen, W J Intracellular po_2 in heart and skeletal muscle *Physiologist* 1971 *14* 69—8..

Whalen W J and *P Nai* Intracellular O_2 and it regulation in resting skeletal muscle of the guinea pig *C rc Res.* 1967 *21* 251— 61

Wiedeman M.P Patterns of the arteriovenous pathways. In *Handbook of Physiol Sect 2* (*Hamilton* and *Dow* Eds.) Vol. II Williams & Wilkins, Baltimore 1963 p. 891—933

W ggers C.J., Physiology of Shock. The Commonwealth Fund. New York. 1950.

Willenkin R.L. and *N.M Greene* Skeletal muscl oxygen tension and metabolism during induced hypotension and during asopressor administration. *A esth.* 1963 *24* 168—176.

Winbury M.M Use of radioisotopic tracers in the study of nutritional circulation. I *Radionuclides in Pharmacology Int Encycl pedia of Pharmacology and Ther peutics.* Sect. *78* Vol. I Pergamon Press. Oxford 1971

Wolpert P W K Noller D Shaughnessy D N Houchen, M.E. Baccari and *F.A Miller* Tissue pH_1 A new clinical tool. *Arch Surg* 1970. *101* 308—313

Wright D.L. and *R.R. Sonnenschein* Relations among activity blood flow and vascular state in skeletal muscle *Am. J Physiol.* 1965 *208* 782—789

Wright J J.H D JJ P.H McLean and *D MacLean* Regional capillary blood flow and oxygen ptake in severe sepsis. *Surg Gyn. Obst* 1971 *132*. 637—644

Yudilevich D.L., E.M Renkin, O.A. Alvarez and *I B avo* Fractional extraction and transcapillary exchange during continuous and instantaneous tracer dministration. *Circ Res.* 1968 *23* 325—336.

Ziegler W.H and *C A Goresky* Transcapillary exchange i the working left ventricle of the dog. *Circ Res 1971 29* 181—208

Zierler K.L., Theory of the use of arterio-venous concentration differences for measuring met bolism in steady and nonsteady states. *J Clin. Invest.* 1961. *40*. 2111—2125

Zierler K.L., Tracer-dilution techniques in the study of microvascular behavior *Fed Proc* 1965 *24* 1085—1091

Zweifach B W Microcirculatory derangement as a basis for the lethal manifestation of experimental shock. *Brit J Anaesth.* 1958 *30* 466—484

Zweifach B W Aspects of comparative physiology of laboratory animals relative to the problem f experimental shock. *Fed Proc* 1961 *20* 18—29

Zwe fach B W and *D.B Metz* Selecti e distribution of blood through the terminal ascular bed of mesenteric structures and skeletal muscle. *Angiol.* 1955 *6* 282—290

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 379

TISSUE HYPEROSMOLALITY AS A MEDIATOR OF VASODILATATION AND TRANSCAPILLARY FLUID FLUX IN EXERCISING SKELETAL MUSCLE

BY

JAN LUNDVALL

LUND 197[illegible]

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 379

FROM THE DEPARTMENT OF PHYSIOLOGY AND BIOPHYSICS,
UNIVERSITY OF LUND
LUND, SWEDEN

ERRATA

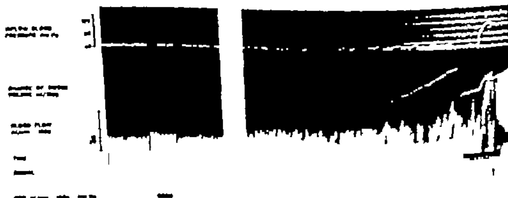


Fig. 38 Intestinal blood flow and tissue volume during "forward" perfusion (panel A) and "retrograde" perfusion (panel B). Retrograde perfusion only possible during reduced inflow pressure. For further explanation see text.

Acta Physiologica Scandinavica suppl. 379
A reproduction of Fig. 38, p. 115 showing the change of tissue volume more clearly than the original print.

Page 93 last line: (see (3)) should be changed to (see (6))
Page 96, line 26 Sentence beginning with "This discrepancy should be changed to "This discrepancy may be explained by functional differences between mesenteric and muscle-capillaries, the mesenteric ones appearing much more permeable (eg [illegible] Pappenheimer 1963)

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 379

FROM THE DEPARTMENT OF PHYSIOLOGY AND BIOPHYSICS,
UNIVERSITY OF LUND
LUND SWEDEN

TISSUE HYPEROSMOLALITY AS A MEDIATOR OF VASODILATATION AND TRANSCAPILLARY FLUID FLUX IN EXERCISING SKELETAL MUSCLE

BY

JAN LUNDVALL

LUND 1972

CONTENTS

CHAPTER IV

CHAPTER V

CHAPTER VI

CHAPTER VII

CHAPTER I

INTRODUCTION

There are few problems in circulatory physiology that have, in spite of intense research, resisted a final solution more than the question of the cause of exercise hyperemia. Experimental evidence strongly suggests that this vital circulatory response is causally linked to muscle metabolism and, further that several metabolic factors contribute to the hyperemia by mutual, synergistic actions (for ref see Barcroft 1963 Mellander and Johansson 1968 Haddy and Scott 1968)

Some years ago the hypothesis was advanced that tissue hyperosmolality produced in working skeletal muscle contributes significantly to the functional hyperemia response (Mellander *et al* 1967) This hypothesis was supported by several later reports of *in vivo* and *in vitro* studies from our laboratories (*e g* Gray *et al*. 1968, Johansson and Jonsson 1968 Mellander and Johansson 1968, Lundvall 1969 Jonsson 1970, Mellander 1970, Mellander and Lundvall 1971)

According to this concept, exercise, through the increased metabolism, leads to formation and release of osmotically active products in muscle thereby raising the osmolality in its interstitial space, *i.e* in the immediate environment of the vascular smooth muscle. This environmental change, in its turn, causes osmotic dehydration of the vascular smooth muscle cells the cell shrinkage *per se* and the consequent changes of transmembrane ionic concentration gradients lead to inhibition of vascular tone and, hence, to the dilator response. The concept implies that an increased interstitial concentration of any particle would contribute to the cell shrinkage and to the vasodilatation provided it does not rapidly penetrate the cell membranes of the vascular smooth muscle.

In the present study this hypothesis will be tested along the following main lines

The magnitude and the time-course of the osmolar changes during graded exercise will be defined and considered in relation to the concomitant vascular reactions. After this, the vascular effects of intra-arterial hypertonic infusions will be analysed when administered at rates producing similar changes of tissue osmolality in the *resting* skeletal muscle to those observed during exercise. The hyperosmolality created by hypertonic infusion to resting muscle will be denoted below as "experimental hyperosmolality" Comparison of the vascular effects in the resistance, precapillary sphincter and capacitance vessels during exercise and experimental hyperosmolality led to a quantitative evaluation of the role of tissue hyperosmolality in exercise hyperemia. These aspects are dealt with in Chapter II which also includes a critical analysis of the experimental approach to the problem. One question that proved important in this connection required

specially designed experiments and is therefore treated separately (Chapter VI). It is concerned with the problem whether during hypertonic infusion vascular smooth muscle is affected directly through the intima of the vascular wall (transintimal effect) or whether the effect is exerted from the interstitial space after transcapillary osmolar distribution.

In Chapter III the resistance function is analysed in greater detail insofar that the resistance changes evoked in very delimited parts of the muscle vascular tree in response to exercise and experimental hyperosmolality are defined.

In Chapter V some aspects of the interaction between the local metabolic and other vascular control systems in skeletal muscle will be considered. This type of study provided some further evidence for the hypothesis that tissue hyperosmolality is involved in exercise vasodilatation The patterns of response to nervous and myogenic control mechanisms within the various consecutive sections of the vascular bed of skeletal muscle are known to be modified in a differentiated way by exercise (Kjellmer 1965 *a* Lundvall *et al* 1967). If tissue hyperosmolality is an important mediator of exercise vasodilatation it is to be expected that it will alter the nervous and myogenic response patterns in the same way as exercise As will be shown, this criterion seems to be fulfilled.

Exercise is known to be associated with a marked net transcapillary fluid movement into the active muscles (*e g* Jacobsson and Kjellmer 1964). This phenomenon does not only lead to a drastic environmental change in the muscle itself in terms of a considerable fluid accumulation, but also can be of great importance for general cardiovascular dynamics since plasma volume becomes reduced. The mechanisms responsible for this capillary fluid transfer are investigated in Chapter IV It will be shown that the fluid transfer can be ascribed mainly to an osmotic process as a result of the tissue hyperosmolality induced by work, although a filtration process as a result of increased capillary pressure contributes to the fluid flux. The processes involved in the fluid disappearance from the muscle after exercise are also investigated in this Chapter

In Chapter VII the results from the present studies and from some others performed in the human being as well as data from *in vitro* studies on vascular smooth muscle are discussed The data taken together strongly support the view that tissue hyperosmolality is an important, but not the sole, mediator of exercise vasodilatation the experimental evidence further indicated that work induced hyperosmolality in the general circulation can establish an important osmolar control of plasma volume during exercise.

CHAPTER II

EFFECTS OF EXERCISE AND "EXPERIMENTAL HYPEROSMOLALITY" ON RESISTANCE, PRECAPILLARY SPHINCTER AND CAPACITANCE VESSELS IN SKELETAL MUSCLE

A quantitative evaluation of the role of tissue hyperosmolality in exercise vasodilatation

In this chapter an attempt will be made to analyse in detail the magnitude and time-course of the changes of tissue osmolality that occur in skeletal muscle during graded exercise and to examine to what extent these changes are correlated to the functional hyperemia response. After this, a quantitative investigation will be performed of the effects on the vascular resistance function in response to "experimental hyperosmolality" in the resting skeletal muscle produced by intra arterial infusion of hypertonic solutions at rates causing similar changes of tissue osmolality to those in exercise. A comparative analysis of the results from these series of experiments permitted an evaluation of the role of tissue hyperosmolality in exercise hyperemia. The pattern of vascular response in the various consecutive sections of the muscle vascular bed to exercise was further reinvestigated and, partly by a different approach than previously used. It was confirmed that work evokes a differentiated pattern of vascular response in the various sections (Kjellmer 1964 *a*) the question whether "experimental hyperosmolality" could evoke the same pattern of vascular response was finally approached.

METHODS

Material and anaesthesia. 112 cats of both sexes, weighing 2 4—5 1 kg (mean value 3 4 kg) were used in this study (part of this material was also used in the studies reported in Chapter IV and V) Anaesthesia was induced with ether after which chloralose (50 mg/kg b w) and urethane (100 mg/kg b.w) was given intravenously Often a small dose of Nembutal (10 mg/kg b w) was added to depress reflex activity during surgery Heparine (3—5 mg/kg b.w) was given before intravascular instrumentation Body temperature was maintained at 37.5 —38.5 C throughout the experiments using a heating pad and a heating lamp

Preparation and experimental techniques The experiments were performed on the acutely denervated vascular bed of the isolated lower leg muscles. The preparation is essentially that described by Kjellmer (1964*a)* In brief the skin was dissected free of the hindleg muscles down to the ankle. The lower leg musc

les were separated from the thigh muscles and the paw was removed at the ankle. Fixation of the tendons of the lower leg muscles permitted essentially isometric muscle contractions during somato-motor fibre stimulation. The bonemarrow cavity of the femur was exposed in its distal part and plugged with cotton soaked in silicone grease to occlude any vascular connections between the femur and the calf muscles. The popliteal artery and vein formed the sole vascular connections between the studied muscle region and the main part of the body In most experiments the muscle preparation was placed in a fixed position inside a water filled plethysmograph (38 C), the skin of the calf deprived of its blood supply being replaced to cover the muscles and also used to seal the proximal entrance of the plethysmograph With this arrangement, tissue volume could be followed continuously with the aid of a well balanced volume piston recorder connected to the plethysmograph. When no plethysmograph was used, the muscles were wrapped in gauze soaked in saline and covered by a plastic sheet to preserve a moist environment. Muscle temperature was then maintained normal using a heating lamp.

The present study includes experiments in which blood flow was free to change ("constant pressure perfusion") and experiments in which blood flow was held constant ("constant flow perfusion") The latter was achieved by the use of a constant perfusion pump (Harvard, Model 1210) and blood flow was then diverted from the ipsilateral femoral artery through the pump to the popliteal artery In both types of flow perfusion, the popliteal vein was cannulated and the venous outflow fram the region shunted *via* tubes to a funnel connected to the right jugular vein. In the constant pressure perfusion experiments and in some of the constant flow experiments an optical drop-recorder unit was inserted in the venous shunt for continuous recording of the blood flow In most of the constant flow experiments the drop-recorder was instead inserted in the shunt on the arterial side. Mean arterial inflow pressure was monitored from a cannula in the contralateral femoral artery during constant pressure perfusion. During constant flow perfusion, it was monitored from a T-tube in the arterial shunt distal to the pump By adjusting the height of the orifice of the outflow tubing from the muscle region, venous outflow pressure could be controlled. In the plethysmographic experiments venous outflow pressure was adjusted so as to produce an isovolumetric state in the resting control period (venous outflow pressure 5—8 mm Hg)

Blood flow arterial pressure and tissue volume were recorded on kymograph paper or on a Grass Polygraph.

In experiments in which intra arterial infusions (or injections) were given, special attention was paid to the problem of thorough mixing with blood In the constant flow experiments this could be achieved by giving the infusions in the tubing proximal to the pump During constant pressure perfusion thorough mixing was accomplished by inserting a specially designed mixing chamber (Fig. 1) in an arterial shunt circuit connecting the ipsilateral femoral artery to the pop-



Fig 1 Schematic drawing of mixing chamber" used to produce turbulence and thereby thorough mixing of blood and intra-arterially infused solutions. The chamber was inserted in a short shunt circuit diverting flow from the femoral to the popliteal artery Scale 1:1

liteal artery The chamber was made of inert plastic material and of silicone tubing Dye infusion test experiments in which this chamber was perfused with water at similar flow rates to those encountered in the *in vivo* experiments showed that by producing turbulence the chamber caused thorough mixing. Mixing was, however highly inadequate without using the chamber Although the viscosity of blood differs from that of water it appears likely that the results of these test experiments are valid for the present *in vivo* experiments. The results below strongly suggest that homogeneous distribution of an infused solution in the muscle vascular bed was only obtained by the use of the mixing chamber and, further that the mixing chamber did not cause aggregation of blood cells or liberation of vasodilator agents in the blood (see Comments) The pressure drop across the mixing chamber was found to be insignificant even at flow rates exceeding those encountered in the present study This was checked in the *in vivo* situation by pressure recordings during blood perfusion at varying rates produced by a pump. The resistance of the whole arterial shunt circuit was similarly found to be quite insignificant.

Muscle exercise (single twitches) was produced by electrical stimulation of the distal end of the severed sciatic nerve using a Grass stimulator model S4 K. Supramaximal stimuli (5V 0 5 msec) at a frequency of 0.25—4 imp/sec were delivered. That the sympathetic fibres are not excited by such stimuli applied for somato-motor fibre activation has previously been shown (*e g* Kjellmer 1964*a*) and was confirmed in the following way The muscles were first shown to contract upon sciatic nerve stimulation and the concomitant exercise hyperemia was observed. When the blood flow hade returned to the control level after work gallamine iodide (Flaxedil) was given intra arterially to block the neuromuscular junction, and the nerve stimulated again as before. No contractions and no blood flow change was now observed showing that the autonomic fibres were not excited. However a further increase of the duration and/or voltage of the stimuli produced a marked increase of vascular resistance, showing that the adrenergic fibres could indeed be activated by stronger stimuli.

In six animals the isometrically developed muscle force during exercise was measured using a force transducer (Grass, Model Ft 10) attached to the Achilles tendon. In these experiments the leg was fixed rigidly by pins inserted into the femur and tibia.

Isotonic and hypertonic (900—1700 mOsm/kg H_2O) solutions were infused into the arterial shunt at rates between 0 1 and 1 9 ml/min using a constant infusion apparatus. Solutions of glucose or sodium lactate were generally used but sucrose, xylose, mannitol urea and NaCl were also tested The solutes were dissolved in distilled water

For measurement of venous and arterial plasma osmolality blood samples (<1.5 ml) were taken from a T tube in the venous outflow tubing close to the popliteal vein and from a catheter in the brachial artery (exercise experiments) or from a T tube in the arterial shunt distal to the mixing chamber (hypertonic infusion experiments) after discarding the dead space" fluid volumes. Such samples were withdrawn before, during and after exercise and hypertonic infusion. After centrifuging, plasma osmolality was determined by thermistor cryos copy (Osmometer 31 LAS, Advanced Instruments, Inc.) Each sample was mea sured twice. When different readings were occasionally obtained the mean va lue was used. Readings in repetitive measurements on osmolar standards devia ted at most by 1.5 mOsm/kg H_2O from the true value. After osmolality had been determined, the sampled blood was returned to the animal. When several blood samples were taken in rapid succession any significant change in blood volume was avoided by the following procedure. A suitable amount of an isotonic dextran-Tyrode s solution was given i.v at least 10 min before the experiment, a time period sufficient for thorough mixing in the general circulation. Concomi tantly a corresponding amount of blood was withheld extracorporeally in the funnel connected to the external jugular vein. By graded return of the extra corporeal blood during subsequent blood sampling the intravascular blood volume was kept approximately constant.

With the present experimental approach it was possible to follow the influence of exercise and of hypertonic infusion on the resistance vessels, precapillary "sphincters and capacitance vessels as well as on net transcapillary fluid movement (for detailed discussion see Mellander 1960 Mellander and Johansson 1968) The resistance function was given by the pressure/flow relation. Changes in precapillary sphincter activity determining the number of patent capillaries and, hence, the functional capillary surface area available for transcapillary exchange, were followed by determination of the capillary filtration coefficient (CFC) It was assumed that no change in capillary permeability occurred in response to exercise or hypertonic infusion (see Comments) CFC was determined by recording the rate of net transcapillary fluid filtration caused by raising venous outflow pressure by a known amount (see Mellander 1960, Cobbold *et al* 1963) For the CFC calculations, it was assumed that 80 % of the venous pres sure rise was transmitted to the capillary level during constant pressure perfusion (*cf* Cobbold *et al* 1963) and 100 % during constant flow perfusion (*cf* Kjellmer 1964 *a*)

The level of vascular tone present in the capacitance (mainly venous) vessels in the control state and during exercise and hypertonic infusion was determined

by observing the rapid tissue volume increase (reflecting changes of regional blood volume: see Mellander 1960, Åblad and Mellander 1963 Mellander and Johansson 1968) caused by a short i.a. infusion of a supramaximal dose of acetylcholine (> 100 μg/kg muscle). This substance is known to cause a virtually maximal dilatation of the muscle capacitance vessels (Åblad and Mellander 1963 Mellander 1966) These experiments were performed under constant flow perfusion to avoid any significant passive capacitance effect due to changed distending pressure in the venous section In some experiments the capacitance effects were recorded concomitantly by external monitoring regional changes in the radioactivity of the red cells labelled with ^{51}Cr (for details see Åblad and Mellander 1963 Kjellmer 1965*a*)

Acetylcholine was also used as a reference substance to induce a short maximal dilatation of the resistance vessels and was given as a slow i.a. injection in a dose exceeding 100 μg/kg muscle (*cf* Kjellmer and Odelram 1965) In order to produce a stable, long-lasting maximal resistance vessel dilatation, papaverine was infused intra arterially (>0 6 mg/min)

For analysis of changes of organic and inorganic constituents of plasma during exercise, arterial and regional venous blood samples were withdrawn as described above. 5 % CO_2 was added to the gas phase of the samples to prevent chloride shift between plasma and erythrocytes. The blood samples were immediately centrifuged and the plasma to be used for determination of organic constituents frozen in liquid nitrogen. The plasma concentrations of lactate, pyruvate and glucose were measured with specific enzymatic (fluorometric) techniques after extraction of the plasma samples at −25 C (see Lowry *et al.* 1964 Goldberg *et al* 1966). The Na^+ K^+ and Ca^{++} concentrations were measured using conventional plasma photometry (Eppendorff) The plasma chloride concentration was measured using the Cotlove chloride analyser (American Instruments Co., USA) pO_2, pCO_2 and pH were determined on blood samples withdrawn anaerobically using microelectrodes (Eschweiler and Co., Kiel, and Radiometer Copenhagen) The plasma bicarbonate concentration was calculated from pCO_2 and pH using the Siggaard Andersen nomogram (1963)

After each plethysmographic experiment (majority of the experiments) the lower leg muscles were dissected free from the bones for determination of muscle tissue weight. The mean weight of the muscles, after correction for any volume changes during the experiments, was 16 9 (±1 4 SD) g/kg b.w In the other experiments this calculated mean value was used to estimate the mass of the studied muscle group from the body weight.

RESULTS

A. Resistance vessels

Attempts to reveal in quantitative terms the causal role of any factor in the functional hyperemia response in skeletal muscle must primarily be directed to an analysis of its effects on the resistance function Experimental approaches aimed at disclosing the mechanisms responsible for exercise hyperemia usually imply studies under conditions that more or less deviate from the normal situation Such circumstances, besides the normal biological variation, can lead to considerably varying results in different experiments. It follows that reliable conclusions may not be reached unless the number of observations is sufficiently large, a demand believed to be satisfied in the present study

The results concerning the resistance vessels will be presented in three sections. In the first two (A1 A2) the general characteristics of the resistance responses and the concomitant osmolar changes during exercise and experimental hyperosmolality will be outlined. An attempt is also made to critically evaluate possible experimental errors. In the last section (A3) the collected data on the resistance changes versus the osmolar changes during exercise and experimental hyperosmolality are presented on a comparative basis to permit an evaluation of the role of hyperosmolality for the exercise hyperemia response.

The plasma osmolality of arterial blood and of the regional venous blood was, in the control period at rest, usually of about the same order of magnitude, the venous value, however sometimes being slightly higher (1—2 mOsm/kg H_2O). Venous plasma osmolality reflecting interstitial osmolality varied to some extent between animals and averaged 318 ± 5 SD mOsm/kg H_2O at the time the surgical procedures were finished.

In the individual animal resting control venous (interstitial) osmolality at times exhibited a slow progressive increase from the initial value in experiments involving several periods of exercise or hypertonic infusion. According to the hypothesis dealt with, an increased interstitial osmolality should elicit vasodilatation through osmotic shrinkage of the vascular smooth muscle. Such shrinkage can be expected to be most clear-cut in response to acute changes of interstitial osmolality but may tend to disappear with time due to solute penetration into the smooth muscle (*cf* Comments) Therefore the effects of more long-termed osmolar changes occurring over a time period of several hours might be negligible. The variation in steady state control osmolality was not found to influence control resistance which is consistent with this view

It appears predictable that a change of vascular smooth muscle volume and a thereby ensuing vasodilatation in response to a given acute increase of interstitial osmolality should be related to the prevailing osmolality in the control period before the experimental intervention. However the variation in control osmolality in the present study was small and such a relation was not revea

led. The vascular effects described below will therefore only be related to the evoked changes of osmolality from the preceding control level absolute figures for total osmolality will thus not be used.

1 Changes of resistance and of plasma osmolality during exercise

The muscles of the lower leg except for the small soleus muscle, mainly consist of "white, phasic" fibres. Although the physiological motor fibre discharge to a muscle like the gastrocnemius can amount intermittently to 50 to 60 impulses per sec, it is well known that a maximal resistance vessel dilatation is elicited at electrical nerve stimulation rates of 4 to 5 impulses per sec (*e g* Kjellmer 1964 *a*, Folkow and Halicka 1968) an observation confirmed in the present investigation. At higher stimulation rates blood flow is impeded owing to mechanical interference of the muscle contraction (*e g* Folkow and Halicka 1968). In work experiments designed to reveal the role of a metabolic factor in exercise hyperemia such low stimulation rates should therefore be used so as to produce single twitches. The experiments were performed both under constant pressure perfusion when, as in the intact organism blood flow is free to change as well as under constant blood flow perfusion In most of the experiments the more physiological condition of constant pressure perfusion was used and these experiments will be described first. For reasons discussed below (Comments section) a dilator response during constant pressure perfusion will be expressed in terms of the evoked change in vascular conductance (flow/pressure) whereas, during constant flow perfusion, it will be expressed as the evoked change of the flow resistance (pressure/flow)

Fig. 2 illustrates an experiment in which the changes in blood flow and in plasma osmolality evoked by exercise at 4 imp/sec were followed In the control period at rest, blood flow was about 13 ml/min x 100 g tissue and arterial and venous osmolality were equal (316 mOsm) It can be seen that blood flow started to increase very shortly after the commencement of exercise and, within some 2 min, it reached about a maximal level of 45 ml/min x 100 g which was then maintained during the period of work. Similarly venous osmolality rose quickly after beginning of exercise, as shown by the blood sample taken after 20—25 sec, and the hyperosmolality reached about a maximal value of 22 mOsm above the control level after some 2.5 min and was then maintained at this level during the period of work. Since arterial osmolality was not altered significantly it is evident that the osmolar change emanated from the exercising muscles, apparently caused by production and release of osmotically active particles by the contracting striated muscle fibres. The change of venous osmolality thus reflects indirectly and with some delay the hyperosmolality created primarily in the extravascular compartments. It follows that hyperosmolality in the interstitial space the immediate environment of the vascular smooth muscle, is established in a very early phase of work. At the cessation of the exercise, both blood flow and venous osmolality gradually returned towards the control levels. Thus expe-

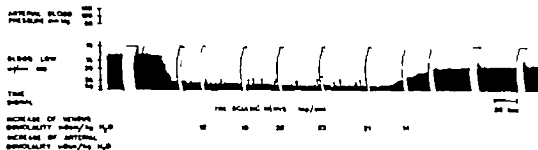


Fig 2 Osmolar changes in venous effluent from cat skeletal muscle during and after short term (~ 5 min) heavy" exercise (somato-motor fibre stimulation 4 imp/sec). Blood flow increased rapidly to reach a maximal level f 45 ml/min×100 g after about 2 min. Venous osmolal ty reflecting interstitial osmolal ty started to rise soon after beginning of exercise and reached a ma imal mai tained increase (~ 2. mOsm/kg H_2O) within 2.5 min. Arterial osmolality irtually unchanged indicating that the venous hyperosmolality emanated from the exercisi g muscle. Upon cessation of exercise, blood flow and venous osmolality returned gradually and in parallel, towards the control levels.

riment strongly suggests that there is a close co-ordination between the osmolar and the vascular changes during and after exercise.

The changes with time of the blood flow and venous osmolality were analy sed in 23 experiments similar to the one described above, but performed at va rying motor nerve stimulation rates (0.25 to 4 imp/sec). Within this frequency range, graded exercise was produced which, with regard to both resistance and osmolar effects, ranged from barely discernible to maximal. For the sake of simplicity exercise evoked by rates less than 1/sec will be classified below as "light" by 1 to 2/sec as "moderate" and by 3 to 4/sec as "heavy" The exer cise hyperemia usually reached a steady state within 60 to 120 sec, the lower figure being more representative for heavy work and the higher more representative for light work. An increased osmolality in the venous effluent was usually observed within less than 30 sec after the commencement of exercise and the hyperosmolality attained a maximal level within 2 to 4 min in the great majority of the experiments. Like the hyperemia response, the venous osmolar change reached a maximum earlier at heavy work than at light work. After exercise, flow and venous osmolality returned to the control levels and largely in parallel The recovery period varied in different experiments but usually did not exceed 20 min. It should be stressed that the osmolar changes in the inter stitium must be more closely co-ordinated in time with the described vascular changes than the above data indicate since the osmolar events in the venous eff luent inevitably lag somewhat behind those in the extravascular space, e g because of the transit time from capillary to the place of venous sampling. Especially in the initial phase of exercise when the hyperemia is not yet developed such time lag must be of significance.

From the experiments described it is clear that, regardless of the work intensity both the changes in vascular resistance and in venous osmolality can be

considered fully developed after 3—4 min of exercise ("steady state phase of exercise") This implies that the question whether there is a quantitative correlation between the hyperemia response and the venous hyperosmolality can be approached by comparing these phenomena during relatively short (<5 min) exercise experiments. Such an analysis was made in 104 experiments performed at varying work intensities Two different experimental procedures were applied. In one type, graded exercise was produced by step-wise increase of the rate of motor nerve stimulation so as to elicit 2 to 3 occasionally 4 consecutive work periods, each of 4 to 5 min duration. In the other the work periods were separated in time so as to permit full recovery of the vascular and osmolar changes in between periods of graded stimulation. No major difference could be found between the results from these two types of experiment.

There was in general, and in particular in the individual experiment, a clear relationship between the rate of motor nerve stimulation (work "intensity") and the extent to which blood flow and venous osmolality rose above the control values in the steady state phase of exercise. During heavy work (4 imp/sec), muscle blood flow was on the average 42 ml/min x 100 g tissue at a perfusion gradient of about 100 mm Hg, but not infrequently it reached some 60 ml/min x 100 g. With few exceptions, the observed flow values at 4 imp/sec represented a maximal, or close to maximal resistance vessel dilatation, as tested by close arterial acetylcholine injection (see Methods) At this stimulation rate, the increase of venous osmolality averaged 22 mOsm, but occasionally amounted to 40 mOsm.

The original record in Fig 3 may serve to illustrate the mentioned relation between the hyperemia and venous hyperosmolality at graded stimulation rates (signal). It can be seen that at a perfusion pressure of about 100 mm Hg (venous outflow pressure 6 mm Hg) blood flow increased step-wise with increasing work intensity from a resting control value of 7.3 to a maximal value at 4 imp/sec of 45.3 ml/min x 100 g tissue. The observed increase of venous osmolality and the calculated changes of vascular conductance in the different exercise periods are shown at bottom of the figure. It can be seen that during exercise these two parameters were closely correlated. In the postexercise period, vascular conductance and venous osmolality gradually waned, and roughly in parallel, down to the control levels, which were reached in about 20 min.

Fig 3 also illustrates the increase of skeletal muscle volume caused by net transcapillary movement of fluid from blood to the active muscles during work. This process will be described in detail below (Chapter IV) One aspect of the transcapillary fluid movement will however be considered here, as it is of relevance for the blood flow determinations. When blood flow as in the present experiments, is recorded as the venous outflow from the muscles, the trancapillary fluid loss implies that blood flow in the precapillary vessels in somewhat underestimated. The rate of fluid movement was related to the stimulation frequency as can be seen in Fig 3 The maximal rate of fluid flux thus occurred at

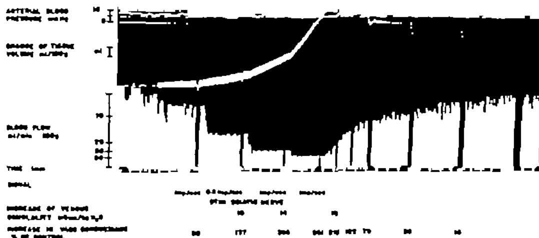


Fig 3. Changes of vascular conductance and venous osmolality during and after graded skeletal muscle exercise Note close correlation between the vascular and osmolar events. Tissue volume curve shows increasing net transcapillary fluid movement into muscle with increasing motor nerve stimulation rate.

4 imp/sec and amounted here to 1 7 ml/min x 100 g tissue, corresponding to 3.8 % of the concomitant venous blood flow (45.3 ml/min x 100 g) At the lower work intensities, this percentage was smaller In none of the exercise periods in which this fluid loss was recorded (as was the case in the majority of the experiments) did it exceed 5 % of the concomitant venous blood flow It follows that during work the total resistance function in the muscle vascular bed can be calculated with quite reasonable accuracy from observations of venous outflow

Collected data from all exercise experiments (n = 104) performed at constant pressure perfusion showed a statistically highly significant correlation between the following parameters 1 stimulation frequency (0.25—4 imp/sec) and observed per cent increase in vascular conductance (r = 0 70 p < 0.001), 2. stimulation frequency and degree of venous hyperosmolality (r = 0 78 p < 0 001) and 3 per cent increase in vascular conductance and degree of venous hyperosmolality (r = 0.51 p < 0 001)

As will be discussed in section A3 the quantitative evaluation of the role of a metabolic factor in exercise hyperemia might be erroneous if the results from experiments are included in the material in which the preparations in the control period exhibited a resistance vessel tone that deviated far from normal. In the processing of data such errors can be avoided by excluding, on a statistical basis, experiments with extreme control resistance values. Fig. 4 is a diagram based on the 83 exercise experiments in which this type of analysis indicated that the resting tone of the resistance vessels could be considered normal (see A3) The increase of vascular conductance, expressed in per cent of the control value, is plotted against the concomitant venous hyperosmolality The

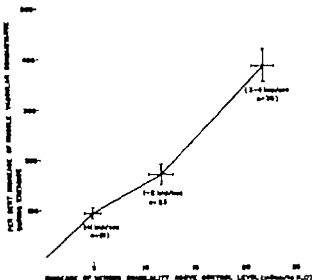


Fig 4 Diagram showing increase of muscle vascular conductance versus the concomitant increase of venous osmolality during graded exercise (n=83) Data, classed with regard to rate of somato-motor fibre stimulation ("work intensity"), refer to observations 2—4 min after commencement of exercise when vascular and osmolar changes were fully developed. Highly significant correlations existed between stimulation frequency and increase of vascular conductance, between stimulation frequency and degree of venous hyperosmolality and between conductance increase and degree of venous hyperosmolality

data are classed with regard to the rate of somato-motor fibre activation and the mean values ± SEM are given. It can be seen that the increase of vascular conductance and the degree of venous hyperosmolality during exercise were related to each other also in this material and that both these parameters, in turn, showed a relation to the stimulation frequency

A possible dilator action of the work induced hyperosmolality on the vascular smooth muscle should be exerted from the interstitial space. As a crude approach to the problem whether the hyperosmolality in this tissue compartment was correctly reflected in the venous effluent the following type of experiment was performed. Heavy muscle exercise was produced (4 imp/sec) and a venous blood sample was taken 3—4 min after the commencement of work, *i.e* in a phase when venous hyperosmolality should have reached its maximal level (see above) Blood flow was then immediately reduced to about 1/10 of that during the hyperemia response with the aid of a screw-clamp placed around the abdominal aorta and repetitive venous blood samples were withdrawn. On average venous hyperosmolality in the phase of reduced flow when the transcapillary osmolar equilibration should be improved as a result of the decreased capillary flow velocity exceeded that in the unrestricted hyperemia phase by 12 per cent (mean of 9 experiments) suggesting that a complete transcapillary osmolar equilibrium may not be established during exercise.

In the human being the post-exercise hyperemia is known to vanish in an approximately exponential manner (Dornhorst and Whelan 1953 Hyman, and Wong 1968) In 39 of the present exercise experiments of varying intensity

in which arterial blood pressure remained constant, the post-exercise excess flow (post-exercise flow minus resting control flow) was plotted semi logarithmically against time. A strictly exponential blood flow decay with a mean halftime (T ½) for flow restoration to the control level of 118 (± 24 SEM) sec, was found in 27 of these experiments. T ½ was not correlated to the degree of exercise hyperemia. The value for T ½ after exercise will be compared with the T ½ value after hypertonic infusion (A 2)

The results hitherto described refer to observations made during exercise of relatively short duration (<5 min) Changes of resistance and of venous osmolality were also studied during longer work periods, usually of 15 min duration which below is denoted "prolonged exercise. During prolonged work, the hyperemia at all stimulation rates usually remained close to its maximal level reached within a few minutes after the commencement of work. At light and moderate exercise, the venous hyperosmolality also generally persisted at its maximal value throughout the prolonged work periods. At heavy work, however venous osmolality showed in virtually all experiments a progressive decline with time from its peak value, but always stayed well above the control level at rest. In 13 exercise experiments at 4 imp/sec, the venous hyperosmolality values after 2 to 4 min and after 15 min of work thus were different ($p<0.001$), the mean increases being 22 ± 2 SEM and 12 ± 3 SEM mOsm, respectively

The declining venous hyperosmolality during heavy exercise seemed to occur in parallel with a gradually decreasing muscle contraction force. The latter phenomenon was attested in a crude way by mere inspection of the contracting muscles and, further by analysis of the amplitude of the twitch recordings as reflected in the muscle volume tracing. To verify these indirect observations, calf muscle isometric tension development during exercise was measured in 6 animals by a force transducer attached to the Achilles tendon. Table I shows the mean changes in vascular conductance, venous osmolality and muscle tension observed in various time periods of prolonged light (0.5 imp/sec) and heavy (4 imp/sec) work. It can be seen that during light exercise the vascular conductance, the osmolality and the muscle tension remained about constant throughout the exercise period once the responses were developed. During heavy work on the other hand venous hyperosmolality and muscle tension after attaining their peak values both showed a gradual decline to significantly lower levels after 15 min whereas no significant reduction of the hyperemia response was revealed. Note, however that venous osmolality after 15 min was still clearly above the control level The drastic decline with time of muscle tension may indicate that exercise produced by nerve stimulation at 4 imp/sec for a *prolonged* period of time represents a "supra-maximal" work which cannot be performed in the intact organism for more than a few minutes. The gradual decrease of venous hyperosmolality during prolonged work at 4 imp/sec, in turn, may therefore possibly be sort of an "experimental artefact"

When, in the same animal a prolonged exercise was repeated after a resting

Time after commencement of exercise:		20 sec	50 sec	3 min	7 min	15 min
Increase of vasc. conductance (% of control)	0.5 imp/sec	23±5	55±4	98±14	123±23	118±30
	4 imp/sec	191±56	303±76	394±59	407±75	416±83
Venous hyperosmolality (mOsm/ /kg H_2O)	0.5 imp/sec	1.0±0.3	1.8±0.5	4.5±0.5	5.4±0.9	6.4±1.4
	4 imp/sec	2.7±0.4	11.7±0.8	22.0±0.9	17.2±1.3	10.2±0.3
Muscle force (g)	0.5 imp/sec	771±195	873±205	911±199	911±196	893±126
	4 imp/sec	863±118	858±122	469±69	285±77	168±58

TABLE I. Mean data ± SEM for vascular conductance increase, venous hyperosmolality and muscle force during prolonged "light" (somato-motor fibre stimulation 0.5 imp/sec) and "heavy" (4 imp/sec) exercise. During light exercise these parameters remained about constant throughout the period of work once the effects were developed. During heavy exercise venous hyperosmolality and muscle force after attaining peak values showed a gradual decline whereas the conductance increase was maintained.

period more than sufficient for recovery of blood flow and of venous osmolality to the control levels, the elicited changes of vascular conductance, venous osmolality and muscle tension were about equally large in the second period of light exercise, whereas the latter two effects were somewhat reduced, but never disappeared in the second period of heavy exercise. The last-mentioned tendency was not revealed in repetitive short-term (<5 min) exercise, nor did the degree of venous hyperosmolality seem to be affected when several sequences of graded brief work evoked in a step-wise fashion were produced.

Arterial osmolality remained largely unchanged at its control level during light exercise even if prolonged. During heavy exercise, however, arterial plasma osmolality increased gradually by some 1—3 mOsm after 3—4 min, and by 3—10 mOsm after 15 min. This increase may mainly be explained by delivery of osmotic products from the contracting lower leg muscles to the general circulation, although, for instance, an increased glucose concentration, not emanating from the active muscles, can contribute (see below). From observations on the

	Rest	4 min of exercise	15 min of exercice
Osmolality mOsm/kg H_2O	321.3 ± 2.3	337.5 ± 7 9 *+16.2*	332.3 ± 4.8 *+11.0*
Na^+ mEq/L	151.3 ± 0.7	158.7 ± 1.2 *+7 4*	153 0 ± 3.5 *+1.7*
K^+ mEq/L	4.3 ± 0.3	6.3 ± 0.5 *+2.0*	5.3 ± 0.7 *+1.0*
Lactate mM/L	1 6 ± 0.6	5 1 ± 1.5 *+3.5*	6.5 ± 1 9 *+4.9*
Glucose mM/L	6 8 ± 0 07	8.6 ± 0 8 *+1.8*	9 8 ± 3 0 *+3.0*
HCO_3^- mEq/L	24 6 ± 1 9	23 8 ± 1.6 *—0.8*	21 9 ± 1 8 *—2.7*
pO_2 mm Hg	41 ± 7	19 ± 5	16 ± 3
pCO_2 mm Hg	56 ± 10	69 ± 11	76 ± 15
pH	7.276 ± 0.065	7 179 ± 0.046	7 103 ± 0.061

TABLE II. Data from analyses on venous effluent from muscle during "heavy" (4 imp/sec) exercise (3 exp.). For description see text.

veno-arterial osmolar difference and the blood (plasma) flow it was apparent that the osmotic delivery was maximal in the time period 2—4 min after onset of exercise, and then amounting to some 0 4—1 0 mOsm/min after which it gradually declined. These observations seem roughly compatible with the figures for arterial hyperosmolality taking into consideration the distribution of the osmolar products primarily in the intravascular compartment and later in extra vascular fluid of inactive tissues.

In order to get some information about the substances responsible for the interstitial hyperosmolality in exercising muscle, the venous plasma concentrations of a number of organic and inorganic substances and venous plasma osmolality were determined in the control period at rest and after about 4 and 15 min of heavy work (4 imp/sec) in three animals. The pO_2 pCO_2, and pH were also measured. Corresponding analyses were performed on arterial blood The following substances were analysed: lactate, pyruvate, glucose, sodium, potassium, calcium, chloride and bicarbonate. Of these substances only those listed in Table II (mean values ± SEM), showed changes of such magnitudes as to be of significance for the concomitant osmolality changes during work. The changes of pO_2, pCO_2 and pH are also shown and these data indicate that the work, which on average increased flow from a control value of 7 to a maintained level of 36 ml/min × 100 g, was quite "heavy" Four minutes after the commencement of work venous plasma osmolality was raised by 16 mOsm/kg H_2O Concomitantly the plasma concentration of sodium was increased by 7 4 mEq/lit, potassium by 2.0 mEq/lit, lactate by 3.5 mM/lit, and glucose by 1 8 mM/lit. The bicarbonate concentration, on the other hand, was reduced by 0.8 mEq/lit. The sum of these concentration changes would, in approximate terms, correspond to an osmolality increase of about 14 mOsm, *i.e* to almost 90 % of the observed osmolar change; (since the osmotic coefficients of the substances are below unity this figure, however represents an overestimation).Sodium and lactate seem mainly responsible for the venous (interstitial) osmolality increase. Glucose was the only of these substances that was significantly increased in the arterial blood and the changes of the others emanated from the exercising muscle. The increase of sodium tentatively can be ascribed to a passive effect of fluid (water) flux from the interstitial space into the contracting skeletal muscle cells (*cf* Chapter IV).

After 15 min of work the osmolality increase averaged 11 mOsm Simultaneously the concentration of sodium was increased by 1 7 mEq/lit, potassium by 1.0 mEq/lit, lactate by 4 9 mM/lit, and glucose by 3 0 mM/lit. The bicarbonate concentration was reduced by 2.7 mEq/lit. The sum of these concentration changes corresponds to an osmolar increase of some 8 mOsm. These figures suggest that in later phases of "prolonged heavy" work, lactate was of special importance for the evoked osmolar change.

The experiments (n = 59) in which blood flow was held constant during exercise supported in essential parts the results arrived at in the "constant perfusion" studies, although some quantitative differences were present. Graded exercise thus led to graded dilatation of the resistance vessels (drop in perfusion pressure) and to graded venous hyperosmolality There was, however relatively little difference in the resistance response at stimulation rates between 2 and 4 imp/sec, whereas the venous hyperosmolality was always greater at the latter rate of stimulation. This difference may be explained by the fact that maximal dilator responses in the resistance vessels are difficult to reveal by the constant flow method (see Comments) At a given stimulation rate the osmolar change

in venous blood was developed somewhat earlier and tended to reach higher levels than in the experiments with constant pressure Thus, at 4 imp/sec, venous osmolality could rise to values above 40 mOsm, the maximal value being 47 mOsm/kg H_2O During prolonged work the hyperosmolality was found to be better maintained under constant flow perfusion than under constant pressure perfusion a difference which partly might be related to a greater wash-out of osmolar products from the muscle during the latter type of perfusion. After 10 min of heavy work under constant flow the venous hyperosmolality thus roughly equalled that observed 2—3 min after the commencement of exercise (n=6)

2 Changes of resistance evoked by experimental hyperosmolality in resting skeletal muscle

The results presented in the previous section strongly indicated that the degree of hyperosmolality created in the extravascular space of muscle during work is correlated to the magnitude of the exercise hyperemia response hyperosmolality thus fulfills a prime criterion of a metabolic factor involved in this reaction In order to analyse in quantitative terms the effects of hyperosmolality *per se* on the resistance function, hypertonic solutions were infused close arterially to the resting skeletal muscle so as to produce an "experimental hyperosmolality" of the tissue in the range encountered in exercise. The osmolar changes during exercise were found to develop rapidly and were fully established within a few minutes after the commencement of work. The analysis of the resistance effects of experimental hyperosmolality should be performed in such a way that these osmolar changes are resembled with regard to magnitude and time course in order to be relevant for the present problem, the role of hyperosmolality in exercise hyperemia. The main emphasis will therefore be placed on results obtained in short term hypertonic infusion experiments in which relatively rapid changes of tissue osmolality were produced.

Tissue hyperosmolality during hypertonic infusion evidently is accomplished *via* transcapillary osmotic exchange, but, as for the exercise experiments in the *previous section, the degree of the induced osmolar change in the extravascular* space cannot be determined directly but merely as it is reflected in the venous effluent. The hyperosmolality of venous plasma probably exceeds that created in the tissue during short term hypertonic infusion (see below) It seemed necessary therefore, to study the vascular effects over a venous hyperosmolality range exceeding that observed in exercise. Hypertonic solutions of glucose or sodium lactate were mainly used, but sucrose, sodium chloride, urea, mannitol and xylose solutions were also tested *i.e* both metabolizable and non-metabolizable substances. The use of sodium lactate was prompted by the finding that venous hyperosmolality of exercise could mainly be attributed to increased concentrations of the sodium and lactate ions (Table II) The effects to be described can be considered as caused by changes of osmolality *per se* and not by more specific actions on the vasculature of the solutes used, since routine control infusions of isotonic



Fig 5 Blood flow changes produced in resting skeletal muscle by intra-arterial infusion of hypertonic sodium lactate solution — (creating experimental hyperosmolality") Flow in creased rapidly to a peak alue and then decreased during continued infusion to attain an pproximate steady level of hyperemia. The observed increase of venous osmolality above control level during the peak flow response and the "steady state flow response is shown. After cessation f infusion flow and enous osmolality returned to control levels in parallel.

solutions obviously creating concentrations in the tissue of the respective solutes far above normal, gave no significant vascular effects. These isotonic infusions, performed at the same rates as the hypertonic ones, also served as control experiments to test the rheological effects of the fluid infusion *per se* These became significant only at the higher infusions rates, and it should be stressed that they have been corrected for in all diagrams below depicting the resistance effects of experimental hyperosmolality

The resistance effects of hypertonic infusion observed both during constant pressure perfusion and constant flow perfusion will be described in relation to the induced venous hyperosmolality As in the exercise section above, the general characteristics of the response as well as some quantitative aspects will be considered first. After this, a critial analysis of possible mechanisms behind these resistance effects will be presented which indicates that they can almost entirely be ascribed to a direct effect on the vasculature in terms of an active inhibitory smooth muscle response.

The general characteristics of the vascular response to experimental hyperosmolality were similar for all substances used, except for urea. The effects of urea will therefore be considered separately later on.

Fig. 5 illustrates the most commonly observed pattern of blood flow response during experimental hyperosmolality in this case caused by sodium lactate in fusion. The pressure head was approximately constant (about 110 mm Hg) throughout the experiment. Blood flow was about 12 ml/min x 100 g tissue in the control period and it increased shortly after the commencement of the hyper tonic infusion to reach a maximal value of about 36 ml/min x 100 g within some 20 sec, at which time venous osmolality had risen by 16 mOsm/kg H_2O. Below

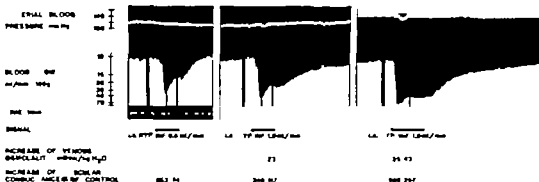


Fig 6. Vascular conductance changes in response to graded experimental hyperosmolality" in resting skeletal muscle

this flow effect will be denoted the "peak response" Blood flow then showed a clear tendency to decrease and it attained an approximate steady state hyperemia level (23 ml/min x 100 g) about 60 sec after the commencement of the flow response, and this hyperemia was maintained during the rest of the infusion period (below denoted "steady state response") Venous osmolality increased by 19 mOsm in the early phase of the steady state response and then showed some further increase. Upon cessation of the hypertonic infusion blood flow and venous osmolality waned gradually and in parallel towards the control values.

In the vast majority of 160 similar experiments in which the hypertonic infusion rate was varied so as to produce graded levels of venous hyperosmolality a similar flow pattern was observed. In this material, (urea infusions not included see below), the peak flow was reached within 15 to 50 sec after the commencement of the response and the steady state flow after another 30 to 90 sec. The flow changes tended to appear earlier at high than at low levels of venous hyperosmolality In some 10 per cent of these short term infusions experiments, especially when either quite small or quite large changes of venous osmolality were evoked, the flow pattern deviated to some extent from the one described Thus, no clear initial "peak response" was seen, but blood flow tended to directly attain an approximately steady state level.

The quantitative relation between the venous hyperosmolality and the concomitant hyperemia response was analysed in these short term hypertonic infusion experiments with regard to changes evoked at the peak response and in the early phase of the steady state response. In the individual animal, repetitive infusions were usually performed, care being taken that full recovery of the vascular response was established in between consecutive infusions A correlation was found between the evoked changes of vascular conductance and of venous osmolality both during the peak response and during the steady state response This is exemplified by the experiment shown in Fig 6 in which sodium lactate was infused at three different rates. During the peak flow responses, vascular conduc

tance increased by 253 340, and 560 per cent and venous osmolality by 9 14 and 35 mOsm the corresponding figures during the steady state responses were 74 117 and 297 per cent and 15 23 and 43 mOsm respectively In both cases there is a close correlation between the vascular and the osmolar changes. It may be pointed out that the peak response shown in the last panel of Fig. 6 represented a virtually maximal resistance vessel dilatation (flow increased from about 11 to about 60 ml/min x 100 g) as checked by a later supramaximal acetylcholine injection.

The bulk of data on osmolar changes during hypertonic infusion was obtained during the steady state vascular response (n = 160) In 61 of these infusions, observations during the peak response were also made. The collected data showed a correlation between the evoked increase of vascular conductance and the con comitant venous hyperosmolality both at the peak response (r = 0.54 $p<0.001$) and at the steady state response (r = 0 70, $p<0\ 001$) During the peak response, a maximal dilatation of the resistance vessels (checked by acetylcholine injection) was not infrequently present when venous osmolality was increased by 20 to 30 mOsm. At this level of venous hyperosmolality there was usually some recovery of vascular tone after the peak response (steady state response) but at higher osmolality levels (40 to 100 mOsm) such recovery was often virtually absent indicating a maintained approximately maximal dilatation of the resistance ves sels.

The question whether the dilator potency was different for the tested substances was analysed only for glucose and sodium lactate and only with regard to the steady state response. These substances were the ones tested in by far the largest number of experiments and the steady state observations provided a suffi ciently large material for such an analysis. In the experiments in which vascular tone in the control period could be considered normal (see section A 3), linear regression analysis relating the evoked per cent increase of vascular conductance to the concomitant venous hyperosmolality gave the following equations for glucose and sodium lactate respectively: $y = 4\ 1x + 7\ 7$ (n = 81 r = 0.80 $p<0.001$) and $y = 5\ 9x - 11.2$ (n = 40 r = 0 88 $p<0\ 001$) Statistical ana lysis, in which the differences of the regression coefficients and of the intersects with the vertical axis were considered simultaneously (Scheffe s method, *cf* Mil ler 1966), showed that above a venous hyperosmolality of 28 mOsm the sodium lactate dilator effect was significantly ($p<0\ 05$) greater than the glucose effect.

During constant pressure perfusion blood flow in the postinfusion period, like after exercise, usually showed an exponential decline. Thus, a strictly mono-exponential decay was present in 29 out of 44 semi-logarithmic plots of excess flow against time. The mean halftime (T½) for the return of blood flow to the control level was for these 29 infusion experiments 86 (± 15 SEM) sec. This value is not different ($p>0.05$) from the corresponding one calcultated for the post-exercise period (A 1) suggesting that the rate, and perhaps the mode, of elimination of the dilator factors after exercise and after hypertonic infusion

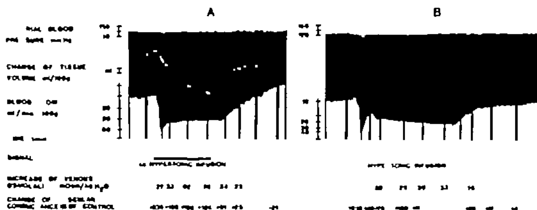


Fig 7 Patterns of blood flow response during "prolonged" hypertonic infusion to resting skeletal muscle. Panel A (glucose infusion) shows the most commonly observed flow pattern, an initial peak and then a less pronounced but maintained hyperemic level despite a gradual rise of venous osmolality Net transcapillary osmotic fluid absorption (tissue volume decrease) caused by the hypertonic infusion also shown. Panel B (sodium lactate infusion) illustrates how after the initial partial recovery from the peak flow response, blood flow sometimes increased parallel to the increasing venous hyperosmolality

were roughly similar (*cf* Dornhorst and Whelan 1953 Mellander and Johansson 1968). Like in exercise, T ½ was not correlated to the degree of the preceding vasodilatation of the resistance vessels.

Fig. 7 illustrates changes of vascular conductance and venous osmolality during more prolonged (7—10 min) infusion periods. Panel A depicts the most commonly observed pattern of response: After the peak response flow (and vascular conductance) remained at approximately constant levels throughout the infusion period, whereas venous plasma osmolality gradually increased with time. Sometimes (Panel B) the partial return of blood flow after the peak response was followed by a successive flow increase parallel to the rising osmolality *After cessation of the infusions, blood flow and venous osmolality in both* cases gradually returned towards the control levels. Panel A also shows the changes of tissue volume caused by hypertonic infusion reflecting net transcapillary fluxes of fluid. During the infusion, an osmotic withdrawal of extravascular fluid to the intravascular compartment occurred and this fluid volume was drained from the region *via* the venous system. It can be seen that the rate of fluid absorption was most rapid in the initial phase of the infusion and then tended to decline with time. Upon cessation of the infusion, fluid gradually returned to the tissue. The extent to which the fluid absorption during hypertonic infusion influenced the calculations of the resistance changes will be considered below It should finally be mentioned that prolonged hypertonic infusion sometimes induced an oscillatory flow pattern with minor fluctuations around a clearly increased mean flow level

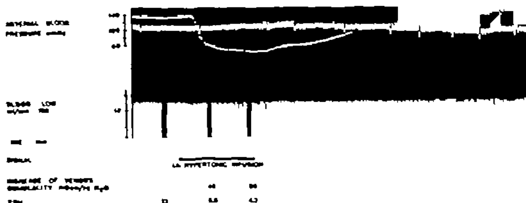


Fig 8 Typical pattern of vascular resistance change in response to intra-arterial hypertonic infusion to calf muscl perfused at constant blood flow Arterial blood pressure (resistance) showed an initial rapid decrease followed by a slower decline towards a steady state level. Note that there was no partial recovery of the hyperosmolar dilator response during constant flow perfusion.

The changes of vascular resistance caused by i.a. hypertonic infusion were studied in 55 constant flow perfusion experiments. The resistance effects were mainly observed after glucose and sodium lactate infusions and they were quite similar whereas some experiments with urea showed a different response (see below). Fig 8 illustrates the typical changes of resistance (perfusion pressure) observed with the two first-mentioned substances. It can be seen that hypertonic infusion (glucose) led to a rapid fall of arterial perfusion pressure within the first minute, after which a further more gradual decline followed. The initial rapid pressure drop corresponded to a regional vascular resistance decrease down to 45 per cent of control resistance which was 13 0 mm Hg/(ml/min x 100 g tissue) The concomitant rise of venous osmolality was 46 mOsm. The venous hyperosmolality increased further to 69 mOsm during the later phase of infusion where resistance had fallen to 4.3 mm Hg/(ml/min x 100 g), *i e* to 33 per cent of the control value. This implied a virtually maximal vasodilatation since a later administered supramaximal dose of acetylcholine decreased resistance to 3 8 mm Hg/(ml/min x 100 g) After cessation of the hypertonic infusion perfusion pressure slowly increased towards the control level which was reached within some 20 min. The control infusion of isotonic glucose solution in this experiment lowered vascular resistance by only 4 per cent indicating that the main resistance effect described was caused by the hyperosmolality *per se*

It should be noted that the resistance response to experimental hypertonicity evoked during constant flow perfusion diverged from the typical response during constant pressure perfusion insofar as the recovery of vascular tone (steady state response) observed with the latter technique was not present. This discrepancy will be considered below (Comments)

The resistance response induced by i.a. infusion of hypertonic urea solution differed from that described for the other solutes. During constant pressure perfusion a rapid and sometimes pronounced increase in blood flow was seen in the initial phase of the infusion but, despite continued infusion, flow then returned slowly towards the control level while exhibiting pronounced oscillations. When the infusion was stopped the resistance vessels constricted further and quite rapidly as shown by the fact that blood flow was considerably less than in the control period before the infusion. Some 2 min later flow increased again to the control level During constant flow perfusion hypertonic urea infusion evoked a similar transient dilator response but the oscillatory phenomenon and the post infusion constriction were less pronounced.

Critical evaluation of the blood flow effects observed during hypertonic infusion The fluid volume absorbed from the lower leg muscles during hypertonic infusion (*cf* Fig. 7 Panel A) deserves consideration when calculating resistance effects since it forms part of the venous effluent. In the experiments performed with constant pressure perfusion, blood flow was recorded on the venous side and hence the fluid absorption implies that postcapillary flow has been correctly estimated whereas precapillary flow has been somewhat overestimated, leading in turn, to an overestimation of the dilator response. The degree of fluid absorption varied with the induced change of osmolality and was also as may be expected, related to the evoked hyperemia (*cf* Chapter IV) Further it was observed that the change of tissue volume was less pronounced during urea infusion than during infusion of the other solutes which in all probability can be explained by differences in the magnitude of the transcapillary osmotic reflection coefficient for the various solutes. The extent to which venous flow exceeds arterial flow can be determined from the recorded change of tissue volume. In the experiment depicted in Fig. 7 (glucose infusion) for example, the fluid absorption amounted to 5.0 ml/min x 100 g during the peak blood flow *i.e* to about 8 per cent of the total venous effluent, and to 2 4 ml/min x 100 g during the early phase of the "steady state flow period" *i.e* to about 5 per cent of the total venous outflow Similar calculations were made from recordings in 66 infusion periods in which the different solutes, except urea, were used. A considerable spread of data was found but mean values of the fluid absorption amounted to 11 (±4 7 SD) per cent and to 7 (±3 4 SD) per cent of the concomitant venous outflow during the peak response and in the early phase of the steady state flow response respectively When relating the fluid absorption to the evoked *increase* of venous blood flow the corresponding figures were 19 (± 8 8) per cent and 15 (± 8.3) per cent. To assess the overestimation of the resistance effect of experimental hyperosmolality when judged from the evoked changes of venous blood flow only the pre-/postcapillary resistance ratio during infusion also has to be known. Assuming this ratio, as an approximate mean value, to be 3 1 the recorded outflow values during the peak response should be corrected by 8 per cent and during the steady state response by 5 per

cent whereas the calculated per cent increase in vascular conductance should be corrected by 14 per cent and 11 per cent respectively Such corrections, it should be stressed, have been made in the diagrams below depicting the dilator effect of experimental hyperosmolality (Figs. 9 and 10) During constant flow perfusion the drop chamber generally was placed on the arterial side and consequently precapillary flow was correctly obtained whereas the fluid absorption during hypertonic infusion implied that postcapillary flow was somewhat underestimated. Therefore total vascular resistance during infusion, in turn, has been overestimated to some extent, i.e the effect of experimental hyperosmolality has been somewhat underestimated Since, however the major part of total vascular resistance is located in the arterial vessels and since the blood flow in these vessels was measured correctly the error during constant flow perfusion will be small and has not been corrected for below (Fig. 11)

Possible rheological effects of the absorbed extravascular fluid during hypertonic infusion in capillary and postcapillary vessels were considered indirectly by intra-arterial infusions of isotonic solutions at similar rates. At the higher rates of infusion some increases of blood flow were noted. Since these effects were comparatively small and since the i.a. infusions affected the blood also on the arterial side of the vascular bed it can be concluded that rheological effects of transcapillary fluid absorption must have been quite insignificant.

During exercise the action of hyperosmolality on the vascular smooth muscle evidently is exerted from the extravascular (interstitial) space. This also seems to be the case during experimental hyperosmolality caused by i.a. hypertonic infusion as indicated by the experiments to be described in Chapter VI It follows that if the barrier between the interstitial space and capillary blood prevents the establishment of an osmolar equilibrium during short-term hypertonic infusion, the extent of extravascular hyperosmolality will be overestimated when determined from samples of the venous effluent. To test whether venous osmolality exceeded the extravascular osmolality during hypertonic infusion the following experiments were designed. The muscle was perfused from a reservoir of arterial blood kept at a constant hyperosmolar level. Repetitive venous blood samples were withdrawn in the phase of induced hyperemia and in a second phase when blood flow was mechanically reduced to about 1/10 of that during the hyperemia response. The samples from the phase when capillary flow velocity was thus reduced, and hence favouring the capillary osmolar exchange, showed significantly lower osmolality values than those taken during the hyperemia phase. This was found in all infusion experiments of the type just described (n = 12) regardless of the extent of the initially produced hyperemia. The hyperosmolality of the venous samples withdrawn during reduced flow averaged 74 per cent of that during the hyperemia phase which strongly indicates that on osmolar equilibrium between capillary blood and tissue is not established during short term hypertonic infusion experiments. Since, as mentioned the interstitial hyperosmolality in all probability is the prime determinant

of the vascular response, the described experiments suggest that the dilator effect of experimental hyperosmolality may be considerably underestimated when related to the osmolar changes in the venous effluent. Such an effect of incomplete transcapillary osmolar equilibrium during hypertonic infusion has not been corrected for. Therefore, the dilator effects to be described (Figs. 9 and 10) may actually have been caused by osmolar changes in the interstitial space that were some 25 per cent lower than in the venous blood.

The described flow increases of hypertonic infusion might possibly, to some extent, be due to effects other than direct inhibition of vascular tone on an osmotic basis. For instance, hypertonic infusion might cause an osmotic dehydration of the vascular walls (which theoretically might increase the vascular lumina), lead to rheological effects by osmotic shrinkage of the blood cells, or cause indirect dilator effects *via* release of dilator agents in the blood or from remote organs (*e.g.* the adrenals). These possibilities seem to be refuted by the following experiments.

In five animals the vascular bed was dilated maximally by i.a. continuous infusion of a supramaximal dose of papaverine. An isotonic infusion, later shifted to a hypertonic infusion of the same rate, was then superimposed, the latter raising venous osmolality to levels exceeding the maximal ones produced in the experiments decribed above (>75 mOsm). In none of these experiments (n = 10) did venous outflow during hypertonicity increase by more than what could be explained by the concomitantly observed net transcapillary fluid absorption (mean increase being 7 per cent). These findings indicate that neither dehydration of the vascular walls nor shrinkage of the blood cells were phenomena of significance for the described hyperemia effects of experimental hyperosmolality.

The following type of experiment served to illustrate that hypertonicity in all probability did not cause a release or production of dilator agents in the blood itself. Samples of blood were made hypertonic up to a level of 700—800 mOsm by the addition of glucose implying that the hyperosmolality considerably exceeded that produced in the hypertonic infusion experiments. Plasma obtained from such pretreated samples was, after dilution to isotonicity, infused intra arterially to the muscles but failed to induce a vasodilatation. It should be stressed in this connection that no macroscopic sign of hemolysis was ever noted in the plasma samples withdrawn in the hypertonic infusion experiments.

β-receptor blockade of the vascular bed (Propranolol) did not alter the magnitude of the hyperemia effects of experimental hyperosmolality. Apparently hypertonicity did not cause a local β-receptor stimulation nor could the effects be ascribed to possible release of adrenaline from the adrenals, the latter conclusion was corroborated by experiments in which both these glands were ligated. The fact that the flow effects of hypertonic infusion was fully developed within a time period usually shorter than that required for recirculation indicated that they were not caused by dilator factors released from re-

mote organs. The described flow effects were not altered by dibenzyline or atropine treatment showing that hypertonicity did not interfere with local α-adrenergic or cholinergic mechanisms in the present experiments.

Although unlikely the possibility exists that the observed flow effects to hypertonic infusion to some slight extent could be related to a change of pH since the infused solutions were not buffered to the pH of blood prevailing in the control period. However any such influence was indirectly taken into account *via* the control isotonic infusions. Furthermore, measurements of venous blood pH during hypertonic infusions at high rates (glucose or sodium lactate) showed that only small and inconsistent changes were produced. On the average, venous blood pH in these experiments (n=8) was decreased by 0.007 units during the peak flow response and by 0.013 units after 5 min infusion.

The flow pattern in terms of a peak and a steady state response, observed during experimental hyperosmolality at constant pressure perfusion, indicates that there is some recovery of vascular tone in the latter phase. The phenomenon could only to a minor extent be ascribed to different rates of fluid absorption in the two phases of the response and was furthermore present in experiments in which muscle blood flow was recorded on the arterial side. A possible explanation for this phenomenon will be given in Chapter III, *i.e.* that it is caused by a myogenic constrictor reaction, evoked by increased transmural pressure in the small resistance vessels, which counteracts the dilator response in the steady state flow phase. Here, mention should only be made that some findings in the present series of experiment supported this hypothesis. Thus, it was shown that if the stimulus for such a myogenic reaction, *i.e.* an increase of small artery pressure during hypertonic infusion, was reduced by mechanical lowering of arterial inflow pressure, the distinction between a peak and a steady state response usually waned significantly or even disappeared. Furthermore, recovery of vascular tone tended to disappear when the muscle was perfused with cold (28 C) blood, a procedure concidered to lessen or abolish myogenic reactivity (*cf.* Lundvall *et al.* 1967).

Another explanation for the peak to steady state flow pattern during hypertonic infusion could be that the peak effect was partially related to a transmural dilator effect on the arterial side as a consequence of an especially high arterial blood osmolality in the earliest phase of the infusion, *i.e.* before blood flow increases. This possibility however seemed refuted by the experiments described in Chapter VI.

3.3 Comparison between dilator effects in resistance vessels evoked by exercise and by experimental hyperosmolality

In a comparative analysis of vascular effects evoked by different experimental procedures, e.g. exercise and experimental hyperosmolality it is important that the control vascular tone is similar in both series of experiments, especially if the effects are expressed in per cent of the control values. This may be illustrated

by the following example: At equal perfusion pressure one dilator factor may be found to increase blood flow in the sympathectomized skeletal muscle from, say *10 to 40 ml/min x 100 g, i.e.* by 300 per cent, and another from 4 to 40 ml/min x 100 g, *i.e.* by 900 per cent. The marked difference in the per cent flow changes may not necessarily represent a corresponding true difference in dilator potency.

Further such a comparative analysis might be influenced by the state of the preparation from which the results are obtained. It cannot be entirely avoided that the anaesthesia, surgery and instrumentation required for detailed circulatory studies on experimental animals sometimes cause some deterioration of the preparation. In consequence, an abnormally low vascular resistance may prevail due to partial loss of normal basal vascular tone or conversely an abnormally raised resistance, caused, for instance, by aggregation of the blood cells. Such signs were occasionally noted also in the present series of experiments. Aggregation *for instance, seemed present in some experiments of long duration* and was revealed as a sudden and maintained decrease of a high resting vascular resistance upon a rapid i.a. injection of saline, apparently causing a washout of sludged cells. A similar washout effect evidently could be caused by exercise and hypertonic infusion under these circumstances, since much larger resistance responses were evoked before than after such a saline injection. Results obtained from deteriorating preparations may thus partially be due to artefacts and should be excluded, if possible.

In an attempt to minimize such possible errors in the quantitative evaluation of the present results, those experiments were excluded which on a statistical basis showed extreme values for control vascular resistance (conductance). For the constant pressure perfusion experiments, the mean value of vascular *conductance* for all control observations (n = 214) before exercise and hypertonic infusion was calculated and for the constant flow perfusion experiments the mean value of vascular *resistance* for all control observations (n = 83) was calculated (note that the number of control observations before exercise was less than the number of experimental observations since graded work often was produced by step-wise increase of the sciatic nerve stimulation rate). Only those experiments were accepted in which control conductance (resistance) did not deviate from the mean value by more than ± 1 SD. In the first mentioned series of experiments the mean control vascular conductance was 0.085 ± 0.032 SD (ml/min x 100 g)/mm Hg. For the constant flow experiments, the mean control vascular resistance was 16.0 ± 7.4 mm Hg/(ml/min x 100 g).

By this processing of the material vascular tone in all the remaining experiments can be considered normal or close to normal for the sympathectomized muscle (see Comments). It implied that 83 out of 104 exercise experiments and 129 out of 160 infusion experiments could be accepted in the series with constant pressure perfusion. For constant flow perfusion the corresponding figures were 45 out of 59 exercise experiments and 40 out of 55 infusion experiments.

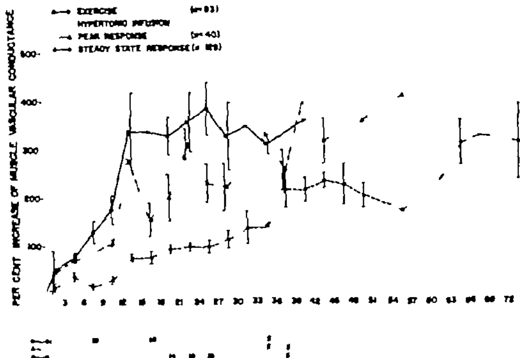


Fig 9 Diagram showing per cent increase of vascular conductance against concomitant increase of venous osmolality above control levels during exercise and during experimental hyperosmolality* in resting muscle. Collected data from constant pressure perfusion experiments. The exercise data (n=83) refer to observations 2—4 min after commencement of work when both vascular and osmolar changes were fully developed. The infusion data refer to the initial peak (n=40) and to the early steady state (n=129) dilator responses. Mean values ± SEM for ± 1.5 mOsm/kg H_2O ranges are shown. Number of observations in each class showed at bottom of the figure.

In the following summarizing section a direct comparison of the effects of exercise and experimental hyperosmolality on the resistance vessels will be made. For the constant pressure perfusion experiments the effects will first be presented as per cent changes of vascular conductance (Fig. 9) Since the hyperemia (increased muscle blood supply) is the main variable during a dilator response of the resistance vessels, the corresponding results are also expressed in terms of absolute values for blood flow (ml/min x 100 g tissue) at a perfusion pressure of about 100 mm Hg (Fig. 10) It should be stressed that in both these diagrams the data refer to true dilator responses since the effects of isotonic control infusions have been taken into account and since, further the data have been corrected for the effects of net transcapillary fluid movements on the venous effluent described in sections A 1 and A 2 above. For the constant flow perfusion experiments the data are presented in terms of per cent changes in regional resistance (Fig. 11)

In Fig. 9 the per cent increases of vascular conductance above the control

values evoked by exercise (open circles) and by experimental hyperosmolality (peak response, triangles steady state response, closed circles) are plotted against the concomitantly observed increases of venous plasma osmolality The diagram shows classed mean values (± SEM) for ± 1.5 mOsm ranges. It should be pointed out that for all three series of experiments mean control vascular conductance was almost the same (0.083 0.086 0.080 (ml/min x 100 g)/mm Hg, respectively) For all three groups of data there was a correlation between the increase of vascular conductance and the degree of venous hyperosmolality (r = 0.56 $p<0.001$ for the exercise data r = 0.61 $p<0.001$ for the peak response data r = 0.81 $p<0.001$ for the steady state response data) The conductance increase during exercise was about maximal (≈ 380 %) at a venous hyperosmolality of about 25 mOsm. The effects during the peak response of experimental hyperosmolality were quite pronounced and often tended to approach those during exercise. On the other hand, with regard to the steady state response, the effects were significantly smaller At a venous osmolality increase of 40 mOsm, *i.e* the upper level during exercise, the conductance increase during the steady state response was approximately 200 per cent. At a still higher osmolality (40—50 mOsm) the conductance changes during the peak response was about equal to the maximal ones during exercise. For the steady state response such pronounced effects was present above 60 mOsm. These latter findings deserve attention *in view of the observation that a transcapillary osmolar equilibrium did not seem* present during hypertonic infusion (section A 2)

The changes in blood flow evoked by exercise and experimental hyperosmolality are shown in Fig. 10 and the data are classed as in the previous figure. Since there was some variation in the perfusion gradient between the individual experiments the comparison of blood flows becomes relevant only if the pressure/flow relationship is taken into account. The average arterial blood pressure in the control period was 108 ± 1.5 SEM mm Hg and venous outflow pressure 5—8 mm Hg, thus giving a mean perfusion pressure close to 100 mm Hg. The average perfusion pressure during exercise and hypertonic infusion was about the same. In an attempt to compensate for pressure effects, all blood flow values have been converted to an arterial pressure of 108 mm Hg, assuming a linear pressure/flow relationship over the actual pressure range encountered. That this assumption was approximately correct was indicated by direct observations in individual experiments of the changes in flow that were elicited by mechanical adjustment of arterial pressure. It can be seen that the relations between the exercise hyperemia and the flow effects during the peak and during the steady state response of experimental hyperosmolality were similar to those in Fig 9 Maximal mean blood flows, about 40 ml/min x 100 g at a perfusion pressure of 100 mm Hg, were reached at a venous hyperosmolality of about 25 mOsm during exercise and of 35—45 mOsm during the peak response. During the steady state response the hyperemia was not too far from maximum above 60 mOsm. A mean blood flow of about 40 ml/min x 100 g is a reasonable average figure for ma

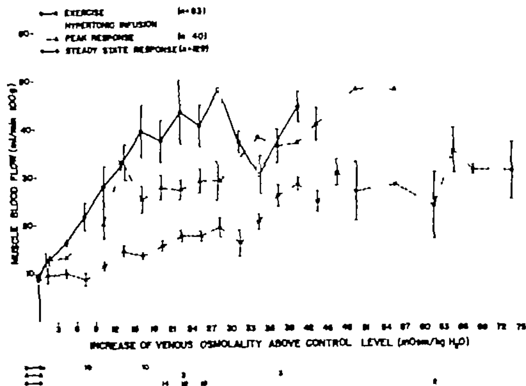


Fig 10 Diagram showing blood flow in skeletal muscle (ml/min × 100 g t a perfusion pressure of 100 mm Hg) during exercise and during experimental hyperosmolality in resting muscle gainst the concomitant increase of enous osmolality Data obtained from same experiments as shown in Fig. 9

ximal blood flow during heavy exercise considering the biological variation (see below). It may be mentioned, however that flow values exceeding 50 ml/min x 100 g were not infrequently observed both during work and experimental hyperosmolality

Fig. 11 shows the resistance responses evoked by exercise (open circles) and by hypertonic infusion (closed circles) during constant flow perfusion Vascular resistance during exercise and during hypertonic infusion is expressed in per cent of the control resistance (100 %) and plotted against the concomitant increase of venous osmolality Mean control resistance was approximately equal in both series of experiments (exercise 17 1 experimental hyperosmolality 16.4 mm Hg/ (ml/min x 100 g)) The diagram shows classed mean values (± SEM) for ± 2.5 mOsm ranges. The data during exercise were obtained at a time period after the commencement of work (2 to 3 min) when the vascular and osmolar changes were approximately fully developed. The data during experimental hyperosmolality were obtained when the initial rapid vasodilator response was

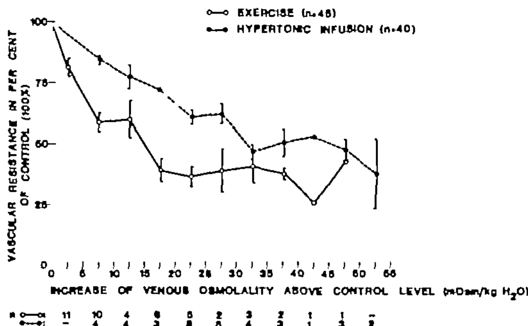


Fig 11 Diagram showing, for constant flow experiments, vascular resistance in skeletal muscle, expressed in per cent of control resistance (100 /), during exercise and experimental hyperosmolality" in resting muscle against the concomitant increase of venous osmolality above control level. Mean values ± SEM for ± 2.5 mOsm/kg H_2O ranges shown. Number of observations in each class shown at bottom of the figure.

established (*cf* Fig. 8) It can be seen that the dilatations elicited by both exer cise and experimental hyperosmolality were related to the degree of venous hyperosmolality (r = —0.70 p<0.001 r = —0 75 p<0 001). In the lower range of venous hyperosmolality (<30 mOsm) the dilatations elicited by exercise were clearly more pronounced than those evoked by hypertonic infusion Above this hyperosmolality level, the response to experimental hyperosmolality approached the exercise effect.

B. Precapillary "sphincters"

The influence of exercise and of experimental hyperosmolality on the precapil lary sphincters was studied by following evoked changes in the capillary filtra tion coefficient (CFC) which reflect alterations in the capillary surface area available for exchange, *i.e* in the number of patent capillaries (see Methods and Comments) CFC was determined by increasing venous outflow pressure by a known amount, hence raising the capillary hydrostatic pressure. It was calcula ted from the observed continuous increase of tissue volume persisting in the pha se where the initial passive distension of the capacitance vessels was fully developed (see Methods) The CFC determination is complicated to some extent by

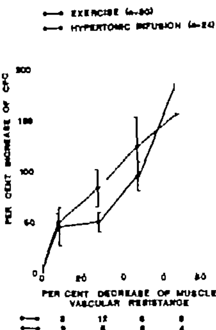


Fig 12 Diagram showing per cent in crease of CFC above control level in the constant flow perfused skeletal muscle during exercise (=30) and during ex perimental hyperosmolality" in resting muscle (=24) gainst the concomitantly observed per cent decrease of vascular resistance below control level. The dia gram shows classed mean values ± SEM. Number of observations in each class shown at bottom f the figure.

the fact that hypertonic infusion and exercise as such evoke net transcapillary fluid movements, mainly by osmosis (section A 1 and A 2 above and Chapter IV). Changes of blood flow will affect the rate of this osmotic fluid flux (Chapter IV), especially during hypertonic infusion because of the consequent changes in arterial blood osmolality (altered dilution of the infused solution in the blood) Therefore, the present analysis of changes in CFC was performed under constant flow perfusion. Even under constant perfusion the osmotic fluxes tend to change with time due to a gradually changing transcapillary osmotic gradient. This implies that the slope of the tissue volume curve before and after the period of raised venous outflow pressure is usually somewhat different. A reasonably correct estimation of CFC could, however be made by calculating the induced fluid filtration from both these "control" slopes and by using the mean of the two values.

In 30 exercise experiments and in 24 hypertonic infusion experiments CFC was determined as soon as the resistance vessel dilatation was fully developed. Control vascular resistance could be considered normal in all these experiments according to the criteria mentioned in section A 3 Mean control CFC before exercise was 0 007 (±0 0005 SEM) and before hypertonic infusion 0.010 (±0.0005 SEM) ml/min x 100 g x mm Hg CFC increased both during exercise and experimental hyperosmolality and roughly in proportion to the extent of the concomitant venous hyperosmolality

The results are summarized in Fig 12 in which the observed increases of CFC are plotted against the concomitant changes in vascular resistance. The data are presented as the per cent increase of CFC above the control level versus the per

cent decrease of regional resistance below the control level (classed mean values ± SEM) There was a correlation between the two responses both during exercise ($p<0.01$) and during experimental hyperosmolality ($p<0.001$) It can be seen that the maximal increase of CFC averaged about 180 per cent during exercise and about 150 per cent during hypertonic infusion These changes of CFC in all probability reflect corresponding increases of the perfused capillary surface area caused by relaxation of the precapillary "sphincters" Experimental hyperosmolality thus seems to influence this section of the vascular bed in similar way to exercise.

C. Capacitance vessels

Reactions within the capacitance vessels can in most situations be followed adequately by recording changes of tissue volume or by external monitoring variations in regional radioactivity of blood labelled with, for instance, ^{51}Cr (*e.g* Mellander 1960 Åblad and Mellander 1963 Kjellmer 1965 *a*) Determination of a capacitance response evoked by exercise or hypertonic infusion by recording of tissue volume is, however made difficult because of the simultaneously evoked net transcapillary fluid fluxes which also contribute to volume changes. Instead, a more indirect approach was used. The vascular smooth muscle tone prevailing in the capacitance vessels in a steady state phase of exercise and of experimental hyperosmolality respectively was compared with that in a preceding control period. The extent of this tone in these different situations could be revealed by observing the acute increase of tissue volume that follows upon a short intra-arterial infusion of a supramaximal dose of acetylcholine known to be capable of bringing the capacitance vessels to virtually complete relaxation (*e g* Mellander 1966) It follows that the greater the response was to acetyl choline, the higher the prevailing tone of the capacitance vessels. Such acute increases of regional blood volume in response to acetylcholine could be recorded accurately by either of the above-mentioned volumetric and tracer methods and the simultaneous use of both these techniques in a pilot series of experiments showed that they both gave quite comparable results. In order to eliminate any significant effect of passive distension of the capacitance vessels resulting from a pressure rise during precapillary dilatation all these observations were obtained during constant flow perfusion The possible mechanical effect on the capacitance vessels *of the muscle contractions per se during work was evaluated* in addition by administering acetylcholine not only during the work but also in the immediate post-exercise period.

Each single experimental evaluation of the influence of exercise or experimental hyperosmolality on the tone of the capacitance vessels was thus based on a comparison of the effects of two acetylcholine infusions, one in the control situation and one during experimental intervention. The latter effect was expressed in per cent of the corresponding effect in the control situation. The present results are based on 16 such paired observations during exercise, 13 obser

vations in the immediate post-exercise period, and 27 observations during hyper tonic infusion. The volumetric method was used in most of these experiments but the ^{51}Cr tracer method was applied in 7 experiments. In all the present ex periments, control vascular resistance fell within a range considered normal (see section A3) The hypertonic infusion rates were adjusted so as to raise venous osmolality by at least 40 mOsm, *i.e* to, or even above, the upper level encountered during work. Work intensity in turn, was adjusted to elicit, on average, a resistance response similar to that during experimental hyperosmolality Thus, vascular resistance (prior to the acetylcholine administration) averaged 55 per cent of control resistance during exercise, 52 per cent in the post-contraction period, and 45 per cent during hypertonic infusion

The mean increase of regional blood volume (capacitance response) evoked by acetylcholine in the control situation was 0.20 (±0.03 SEM) ml/100 g tissue in the exercise experiments and 0.26 (±0.01 SEM) ml/100 g tissue in the infusion experiments. During exercise, in the post-exercise period, and during hypertonic infusion, the capacitance responses to acetylcholine were only slightly less, and averaged 80, 83 and 78 per cent of the control responses, respectively There was no statistical difference between the three groups. These results indicate that both exercise and experimental hyperosmolality caused only little inhibition of the tone in the capacitance vessels despite the fact that the tone of the resistance vessels was markedly decreased When scrutinizing the individual experiments it was found in each of the three groups that the magnitude of this inhibi tory capacitance response tended to become greater the greater the dilatation of the resistance vessels, but even at a maximal resistance vessel dilatation the capa citance vessels were far from a state of complete relaxation. It may be noted that, although the main capacitance function is located on the venous side, a di latation of the resistance vessels (mainly precapillary) leads to some increase of regional blood volume. The experimental data, when taken together therefore suggest that the influence of exercise and experimental hyperosmolality on venous tone may be even smaller than that indicated by the above-mentioned fi gures for the decline of the capacitance response to acetylcholine. Because of the similarity between the results obtained during exercise and in the post-exercise period the conclusion could also be drawn that the muscle contractions caused no significant interference with the capacitance function at the rates of somatomotor fibre stimulation used.

COMMENTS

In this study an attempt was made to examine the role of regional tissue hy perosmolality in exercise hyperemia by analysing on a quantitative comparative basis the reactions within the resistance vessels, precapillary sphincters and capa citance vessels in skeletal muscle (cat lower leg muscles) evoked by exercise and by experimental hyperosmolality the latter produced by intra-arterial hyper tonic infusion to the resting tissue. This investigation is a continuation and ex

tension of a previous study (Mellander *et al* 1967) and it confirms our previous conclusion that tissue hyperosmolality is a mediator in exercise hyperemia.

Pattern of response in consecutive vascular sections In section A1 the characteristics of the changes in osmolality and vascular resistance were examined during graded exercise under both constant pressure and constant flow perfusion. Exercise was found always to be associated with locally produced tissue hyperosmolality the extent of which was estimated to the amount it was reflected in the venous effluent. The hyperosmolality developed almost immediately at the commencement of work and within a few minutes reached a maximal level in the venous effluent. The dilator response of the resistance vessels showed a similar time course, commencing almost immediately upon exercise and reaching a maximal level within 1 to 2 minutes. The extent of venous hyperosmolality when fully developed showed a highly significant positive correlation to the magnitude of the concomitant dilator response of the resistance vessels, both these phenomena, in turn being correlated to the intensity of the work (rate of somato-motor fibre stimulation, 0.25—4 imp/sec). During heavy (4 imp/sec) exercise venous osmolality could exceed that at rest by as much as 40 to 45 mOsm/kg H_2O Venous hyperosmolality remained at the maximal level reached after 2—4 min throughout prolonged periods (15 min) of light and moderate exercise (0.25 to 2 imp/sec) but tended to decline during prolonged heavy work, and then parallel to a declining muscle tension.

When designing experiments aiming at disclosing the role of tissue hyperosmolality *per se* in exercise hyperemia, due attention must be paid to the fact that both the osmolar and vascular changes in work develop rapidly Attempts were therefore made to create by i.a. hypertonic infusion an experimental hyperosmolality in the resting muscle which resembled as closely as possible that in exercise with regard to magnitude and time-course. The hypertonic solutions consisted of non-electrolytes or electrolytes, the latter usually sodium$^+$lactate$^-$since these two ions seemed mainly responsible for the interstitial hyperosmolality of exercise (A1) Since, as in the exercise experiments, changes in tissue osmolality during hypertonic infusion could not be determined directly they were followed as they were reflected in the venous effluent. The results concerning the effects on vascular resistance of experimental hyperosmolality were presented in section A2. Experimental hyperosmolality was always associated with a rapidly developing dilator response of the resistance vessels which, in the constant pressure perfusion experiments, was shown as an initial large flow increase reaching a peak value within less than 50 sec usually followed by a steady state response at which flow stabilized at a somewhat lower level There was a highly significant correlation between the magnitude of the dilator response of the resistance vessels (both the peak and the steady state response) and the concomitant increase of venous osmolality (Figs. 9 and 10) With regard to the peak response, a maximal dilatation of the resistance vessels was present at a rise of venous osmolality by 40—50 mOsm To maintain an approximately maximal dilatation

as a steady state response, venous osmolality usually had to be raised by at least 60 mOsm above the control level. Similar conclusions were reached in the hyper tonic infusion experiments performed under constant flow perfusion with the exception that there were no signs of a secondary partial recovery of resistance during the infusions. It was thus established that resistance and osmolar changes are correlated phenomena, both during exercise and during experimental hyper osmolality Such a correlation was furthermore present during the phase of recovery of vascular tone in the post-exercise and post-infusion periods. The pattern of vascular response during exercise and experimental hyperosmolality was found to be similar not only with regard to the reactions in the resistance vessels but, as shown in section B and C, also in the precapillary "sphincters" (see further below) and in the capacitance vessels. Thus in both situations, the sphincters showed dilatations which increased gradually in relation to the evoked hyperosmolality whereas the tone of the capacitance vessels was relatively little affected, if at all.

Analysis in the post-exercise period suggested that flow returned to the control level in an approximately exponential manner in consonance with the observation by Dornhorst and Whelan (1953) This seemed also true for the flow restoration after hypertonic infusion and with a rate constant roughly similar to that after exercise. Dornhorst and Whelan concluded that if post-exercise hyperemia was related to the local concentration of some metabolite, its removal or destruction could not be critically dependent upon the rate of flow It is therefore likely that elimination of the dilator factor(s) in exercise can depend upon several different processes. With regard to interstitial hyperosmolality it may be abolished after exercise *via* a wash-out to the blood stream, *via* re-uptake of osmolar products into the intra-cellular space of the striated muscle fibres, *via* water transfer into the interstitial space and *via* elimination with the lymph. It is possible that such modes of elimination lead to changes of interstitial hyperosmolality compatible with an exponential flow decay after exercise and also after experimental hyperosmolality

A critical analysis was performed to reveal to what extent mechanisms other than true inhibition of vascular tone on an osmotic basis could have contributed to the changes of the resistance function noted in response to hypertonic infusion. First it should be stressed that rheological effects of the infused fluid *per se* and of the fluid absorbed from the extravascular space due to osmosis were taken into account in the presented diagrams, as well as the extent to which the latter phenomenon influenced the magnitude of the venous effluent. By special experiments the possible involvement of the following factors was analysed: *1* Hypertonic infusion might have caused a passive increase of vascular lumina by dehydration of the vascular walls or a passive resistance decline due to decreased viscosity of the blood as a result of osmotic shrinkage of the erythrocytes (*cf* Overbeck *et al.* 1961) (the latter mechanism being somewhat remote in view of previous *in vitro* findings that the expected decrease in viscosity is counteracted

by a reduced viscous deformability of the osmotically shrunken red cells (*e g* Rand and Lacombe 1964 Meiselman *et al* 1967 Schmid Schönbein and Wells 1969)) *2* The hypertonic solutions could have caused release or production of dilator agents in the blood itself (for instance as a result of hemolysis, see Söderström 1944 however Stainsby and Fregly (1968) reported pronounced vasodilation in muscle to hypertonic infusion when plasma was used as perfusate) or *3* could have caused release of adrenaline from the adrenals or liberation of depressor agents from other tissues *4* The response to hypertonic infusion could have been mediated *via* β-adrenergic or cholinergic mechanisms. The experimental evidence strongly suggested that none of these mechanisms could have played any significant role in the observed effects on the resistance function of hypertonic infusion in the present study It may thus be concluded that the described changes in resistance vessels to experimental hyperosmolality most likely can be ascribed to a direct active inhibition of the vascular smooth muscle tone.

This opinion is strongly supported by a number of studies performed *in vitro* on a smooth muscle preparation, the rat portal vein, which shows functional characteristics similar to those of the single unit type of vascular smooth muscle residing in resistance vessels and precapillary sphincters (Mellander *et al* 1967 Johansson and Ljung 1967 Johansson and Jonsson 1968 Arvill, Johansson and Jonsson 1969 Johansson 1969) These studies, considered in greater detail below (General discussion) showed that, owing to osmotic shrinkage of the effector cells, the spontaneous rhytmical electrical and mechanical activity of the vascular smooth muscle was promptly inhibited when the medium was made hypertonic and this inhibition was sustained when the increased osmolality was due to the addition of substances that did not readily penetrate the cell membranes of the vascular muscle (*e g* sucrose or glucose) Such an effect was not maintained if urea was used which is in consonance with the above described *in vivo* response to this particular substance which quickly penetrates the cell membranes.

The effects observed in precapillary sphincters and capacitance vessels deserve comment. Changes of precapillary sphincter activity were judged from observed alterations of CFC. CFC depends not only on the tone of the precapillary sphincters which in turn, determine the number of patent capillaries and, hence, the capillary surface area available for filtration but also on the specific permeability of the capillary membranes themselves. Any induced change of the latter factor will consequently influence the magnitude of the CFC. By studying the lymph/plasma ratio of dextran fractions of various molecular weights, Arturson and Kjellmer (1964) obtained strong evidence that exercise does not alter capillary permeability Further Landis and Sage (1971) found no evidence for increased capillary permeability when exposing mesenteric capillaries to pronounced hyperosmolality The fact that CFC rose to similar magnitudes during pronounced experimental hyperosmolality and during heavy exercise in the present study can be taken as circumstantial evidence for unaltered capillary permeabi

lity It should then be noted that even a fairly small increase of capillary permeability during experimental hyperosmolality if present, would be expected to have increased CFC clearly above the maximal value during exercise since transcapillary hydrodynamic flow varies with the fourth power of the capillary "pore" radius. It may be concluded that exercise and experimental hyperosmolality could dilate the precapillary sphincters to about the same extent.

The tone of the capacitance vessels was found to be insignificantly affected by exercise as well as by experimental hyperosmolality With regard to exercise, the results confirm an earlier conclusion by Kjellmer (1964 a) who used a somewhat different experimental approach. The present reinvestigation of the capacitance response during exercise was prompted in order to study the possibility that the absence of a marked dilator response in the capacitance vessels might be due to a mechanical influence of the muscle contractions on the capacitance function which might have prevented them from dilating. The results obtained in the post-exercise period, however made this explanation most unlikely

It can thus be concluded that exercise and experimental hyperosmolality both can elicit the same differentiated pattern of vascular response in skeletal muscle· A pronounced dilatation within the resistance vessels and precapillary sphincters, no significant change of capillary permeability and no significant active dilatation of the capacitance vessels. These findings support the view that tissue hyperosmolality can be a mediator of exercise hyperemia.

Some basic aspects of the approach to the comparative study of the resistance function In a quantitative evalution of the role of hyperosmolality in the exercise hyperemia response, emphasis, by definition, must be placed on the effects on the vascular resistance function. The techniques used to study this vascular function and the processing of the present data deserve consideration. The resistance function was analysed under constant pressure perfusion as well as under constant flow perfusion. Some advantages and some disadvantages are inherent in both techniques. The constant pressure perfusion method is, of course the much more physiological one, since as in the intact organism blood flow is free to change. It provides quite accurate information about dilator responses over the entire range of changes in vascular smooth muscle tone, including states of maximal dilatation In that respect the constant flow perfusion method is inferior since, even if small to moderate dilator responses can be adequately followed, pronounced responses tend to become quantitatively underestimated. It is common knowledge, and also noted in the present study that with this method vascular resistance in maximally or close to maximally dilated states hardly ever decreases to the same extent as under constant pressure perfusion. This may be due to abnormal influence of passive factors (e g passive elastic recoil aggregation, or "critical closure") present under these somewhat unphysiological conditions which prevent perfusion pressure from declining to predicted very low levels. Exercise certainly can lead to a maximal resistance vessel dilatation but

by a reduced viscous deformability of the osmotically shrunken red cells (e.g. Rand and Lacombe 1964 Meiselman *et al* 1967 Schmid Schönbein and Wells 1969)) *2* The hypertonic solutions could have caused release or production of dilator agents in the blood itself (for instance as a result of hemolysis, see Söderström 1944 however Stainsby and Fregly (1968) reported pronounced vasodilation in muscle to hypertonic infusion when plasma was used as perfusate) or *3* could have caused release of adrenaline from the adrenals or liberation of depressor agents from other tissues *4* The response to hypertonic infusion could have been mediated *via* β-adrenergic or cholinergic mechanisms. The experimental evidence strongly suggested that none of these mechanisms could have played any significant role in the observed effects on the resistance function of hypertonic infusion in the present study It may thus be concluded that the described changes in resistance vessels to experimental hyperosmolality most likely can be ascribed to a direct active inhibition of the vascular smooth muscle tone.

This opinion is strongly supported by a number of studies performed *in vitro* on a smooth muscle preparation the rat portal vein, which shows functional characteristics similar to those of the single unit type of vascular smooth muscle residing in resistance vessels and precapillary sphincters (Mellander *et al* 1967 Johansson and Ljung 1967 Johansson and Jonsson 1968 Arvill, Johansson and Jonsson 1969 Johansson 1969) These studies, considered in greater detail below (General discussion) showed that, owing to osmotic shrinkage of the effector cells, the spontaneous rhythmical electrical and mechanical activity of the vascular smooth muscle was promptly inhibited when the medium was made hypertonic and this inhibition was sustained when the increased osmolality was due to the addition of substances that did not readily penetrate the cell membranes of the vascular muscle (e g sucrose or glucose). Such an effect was not maintained if urea was used which is in consonance with the above described *in vivo* response to this particular substance which quickly penetrates the cell membranes.

The effects observed in precapillary sphincters and capacitance vessels deserve comment. Changes of precapillary sphincter activity were judged from observed alterations of CFC. CFC depends not only on the tone of the precapillary sphincters which, in turn, determine the number of patent capillaries and hence, the capillary surface area available for filtration, but also on the specific permeability of the capillary membranes themselves. Any induced change of the latter factor will consequently influence the magnitude of the CFC. By studying the lymph/plasma ratio of dextran fractions of various molecular weights, Arturson and Kjellmer (1964) obtained strong evidence that exercise does not alter capillary permeability Further Landis and Sage (1971) found no evidence for increased capillary permeability when exposing mesenteric capillaries to pronounced hyperosmolality The fact that CFC rose to similar magnitudes during pronounced experimental hyperosmolality and during heavy exercise in the present study can be taken as circumstantial evidence for unaltered capillary permeabi-

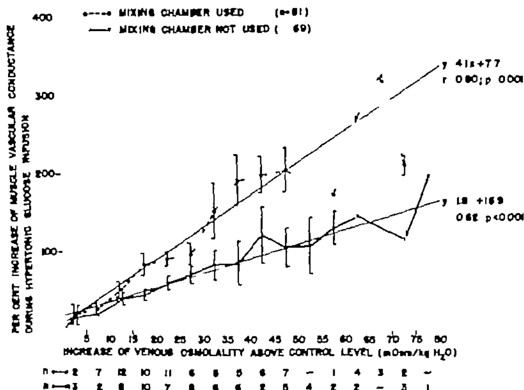


Fig. 13. Diagram showing the ffect of the mixing chamber" (see Fig. 1) on the magnitude of the resistance vessel dilator response to experimental hyperosmolality" (glucose) in resting muscle. The per cent increase of vascular conductance is plotted against the concomitant enous hyperosmolality evoked with (broken line) and without (solid line) use of the mixing chamber Data refer to the early steady state dilator response (mean values ± SEM for ± 2.5 mOsm/kg H_2O ranges). Number of observations in each class indicated. The hyperosmolar dilator response was much more pronounced when the mixing chamber was used (*f* regression coefficients), *an effect ascribed to more homogenous distribution of hypertonic solution in* the muscle.

so as to satisfy within limits, the tissue demand for a supply of nutrients and oxygen and for the removal of waste products. Muscle blood flow is linearly related to the work performed over a wide range of work intensities (e g Kramer *et al.* 1939 Wright and Sonnenschein 1965) At a given rate of motor nerve sti mulation, the muscle contraction force decreases in relation to an inforced limi tation of blood flow (Hirvonen and Sonnenschein 1962) Blood flow is thus of prime importance for skeletal muscle function. Investigations of mechanisms involved in exercise hyperemia should therefore preferably be made under con stant pressure perfusion when flow is free to change. It would also seem most appropriate to express the dilator responses of the resistance vessels to exercise, or to a factor involved in this reaction, in terms of changes of blood flow or of

vascular conductance (flow/pressure) Vascular effects are, however often ex pressed as changes of resistance (pressure/flow) Due to the inverse relation of resistance to flow which is the prime variable, there will be, simply for mathematical reasons, a succesively poorer resolution of the resistance data the greater the vascular response which is not the case using flow or conductance. In a study like this one, in which the vascular effects of a possible mediator in exercise hyperemia are to be compared with those of exercise itself this fact implies that the importance of the factor would appear considerably greater if based on resistance instead of conductance data, as illustrated by the following example. Suppose, for instance, that a constant pressure of 100 mm Hg, such a factor increased blood flow from 10 to 25 and exercise from 10 to 40 ml/min×100 g tissue. This corresponds to a resistance change from 10 to 4 and from 10 to 2.5 units, respectively and a conductance change from 0 10 to 0.25 and from 0 10 to 0 40 units, respectively The factor might then be held responsible for 80 % of the exercise response if calculated from the resistance data, but only for 50 % if derived from the conductance data. This calculation may serve to illustrate the difficulty in evaluating results from different investigations unless due attention is paid to the presentation of the material in terms of resistance or conductance data. In the present study all data from the constant pressure perfusion experiments were expressed in terms of changes of conductance or flow It should be stressed, however that dilator responses obtained in experiments using constant flow perfusion may most appropriately be expressed in terms of resistance changes, since, under these circumstances, resistance varies directly with the recorded variable (perfusion pressure) and this convention was adopted in the present constant flow experiments.

In recent years it has been clearly established that the magnitude of a vascular response, e g a dilator response of the resistance vessels, to a given stimulus is significantly influenced by and often directly related to the level of vascular tone prevailing in the control period prior to the application of the stimulus (e g Myers and Honig 1969) This phenomenon which was observed also in the present study may be related to different mechanisms. The vascular smooth muscle may vary its capacity to respond with its initial degree of stretch, determined for instance by the prevailing vascular transmural pressure, or through a possible effect on smooth muscle membrane potential (Myers and Honig 1969) It might also to some extent be related to more passive, mechanical effects, such as a washout of sludged cells as flow increases. Prior to the stimulus sludging could have created an abnormally high resistance Whatever the case, it must be very important in any quantitative study of vascular effects, especially in a comparative investigation like the present one, to avoid in the evaluation of the results the influence of this phenomenon. In the present study this was done by basing the comparison of the vascular effects of exercise and experimental hyperosmolality on experiments in which the vascular tone prior to experimental intervention was equal (see section A3) Control vascular conductance averaged for

the whole material of constant pressure perfusion experiments 0.085 (ml/min x 100 g tissue)/mm Hg which thus corresponds to an average blood flow of 8.5 ml/min×100 g tissue at a perfusion pressure of 100 mm Hg. These figures are consistent with those reported for other extensive materials concerning resting tone in the acutely symphathectomized white skeletal cat muscle (for ref see Barcroft 1963 Mellander and Johansson 1968) This justifies the conclusion that resting vascular tone in the present experiments could be considered normal for the denervated muscle vascular bed Even under these circumstances, there will of course be some spread of the results due to biological variation etc, which necessitates that the conclusions are based on a sufficiently large material This demand is believed to be satisfied in the present study in which, with regard to resistance effects, the total number of experiments on preparations with normal resting vascular tone was 212 of which 83 refer to exercise experiments and 129 to hypertonic infusion experiments.

Quantitative evaluation of the role of tissue hyperosmolality in exercise hyperemia. The present results show that exercise is associated with a pronounced increase of tissue osmolality This environmental change develops rapidly and in parallel to the functional hyperemia, and it reaches at a given work intensity a maximal level within a few minutes after commencement of exercise To reveal the possible role of tissue hyperosmolality in exercise hyperemia it was considered important that the experiments in which experimental hyperosmolality was produced were designed so as to mimic as closely as possible the osmolar changes during exercise both with regard to magnitude and time-course. This could be done by acute relatively shortlasting hypertonic infusions and emphasis is therefore placed on the dilator responses of the resistance vessels evoked in the early period of the infusions. The diagrams in Fig. 9 and 10 summarized in quantitative terms the vascular conductance and blood flow increases evoked by exercise and experimental hyperosmolality in relation to the concomitantly observed increases of venous osmolality Fig. 11 summarized the corresponding results for the constant flow perfusion experiments. Fig 14 derived from Figs. 10 and 11 serves to illustrate the extent to which tissue hyperosmolality may contribute to the exercise hypermia response. In this diagram the resistance vessel dilator responses during experimental hyperosmolality have been expressed in per cent of the exercise dilator responses at corresponding levels of venous hyperosmolality up to the highest ones encountered in exercise. Thus, for the constant pressuré perfusion data (Fig 10) the *increase* of blood flow above the control level during experimental hyperosmolality has been expressed in per cent of the *increase* of blood flow during exercise for the constant flow data (Fig 11) the *decrease* of vascular resistance (% of control) during experimental hyperosmolality has been expressed in per cent of the corresponding *decrease* evoked by exercise. At any level of venous hyperosmolality the exercise hyperemia response it set to 100 per cent. To facilitate this deduction, the random variation in the results (Figs. 10 and 11) was neglected by drawing by eye a smooth curve for

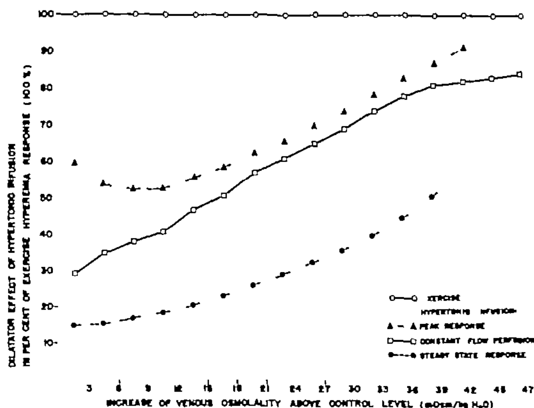


Fig. 14 Diagram showing the extent to which tissue hyperosmolality in working muscle may contribute to the exercise hyperemia response. The resistance vessel dilator response to experimental hyperosmolality in the resting muscle (blood flow increase above control level) is expressed in per cent of the exercise hyperemia response (blood flow increase above control level) at the given levels of observed venous hyperosmolality. The exercise hyperemia response at any level of work induced venous hyperosmolality is set at 100 per cent. The curves refer to the peak and "steady state" dilator effects of experimental hyperosmolality during constant pressure perfusion and to corresponding observations under constant flow perfusion (see key). *Although different, all three curves suggest that tissue hyperosmolality is an important mediator of exercise hyperemia. Which one of the curves is most relevant in this respect is discussed.*

each series of experiment taking into concideration the number of observations in each point. (When constructing the exercise hyperemia curve the fortuitous (?) low blood flow value between 33 and 36 mOsm (Fig. 10) was ignored).

It can be seen from Fig. 14 that for the constant pressure perfusion experiments the peak response during experimental hyperosmolality corresponded to some 50 to 90 per cent of the exercise hyperemia response. The corresponding fraction for the steady state response was considerably smaller varying from some 15 to some 60 per cent. The middle curve illustrates corresponding data for the constant flow perfusion experiments and it shows that the dilator effects

of experimental hyperosmolality under these conditions amounted to some 30 up to some 85 per cent of the exercise hyperemia response.

No doubt the curves in Fig. 14 are different and problems arise in deciding which one best depicts the quantitative role of tissue hyperosmolality in exercise hyperemia under these conditions of short term exercise It would seem that the effect can be no less than shown by the steady state response curve and no more than shown by the peak response curve. Common to all three curves is that they incline with increasing hyperosmolality This can be taken to indicate that the relative role of tissue hyperosmolality in exercise hyperemia is greater the greater the work intensity since, as shown in Fig 4 the degree of work induced hyperosmolality was correlated to the rate of somato-motor fibre stimulation ("work intensity") As mentioned above (A 2) and as will be discussed in Chapter III a counteracting myogenic constrictor effect may be responsible for the partial recovery of vascular tone upon transition from the peak to the steady state dilator response to hypertonic infusion. The stimulus for this myogenic reaction *i.e* a rise of transmural pressure in the smallest precapillary vessels, is not present during exercise nor during the early phase of the peak response and apparently neither during constant flow perfusion when the drop of perfusion pressure should tend to balance an increase of transmural pressure. The fact that the peak response curve and the constant flow curve in Fig 14 are quite similar lends support to this view The mentioned transmural pressure increase may be considered an experimental artefact gradually developing during hypertonic infusion (Chapter III) Circumstantial evidence therefore suggests that the steady state response curve of Fig. 14 may be less valid than the two others for the evaluation of the role of tissue hyperosmolality in exercise hyperemia. The peak response data and the constant flow data, when considered together with *average* values for tissue (venous) hyperosmolality induced by exercise (Fig. 4) suggest that more than 40 per cent of the exercise hyperemia can be ascribed to tissue hyperosmolality during light short-term exercise and more than 60 per cent during heavy short-term exercise. Whichever of the curves in Fig. 14 is used for the evaluation, however the data strongly suggest that tissue hyperosmolality is an important mediator of functional hyperemia in muscle. This opinion is strengthened by the observation that a transcapillary osmolar equilibrium may not be present during exercise and hypertonic infusion (A 1 A 2, Chapter IV) Such a phenomenon, as mentioned (*e g* A 3), would lead to an underestimation of the role of hyperosmolality in exercise hyperemia with the present approach to the problem and it was not corrected for in the data of Fig. 14

The data presented above (A 1) showed that tissue hyperosmolality was maintained during prolonged "light" and "moderate" work. During "heavy" work, however it decreased with time but then in parallel with a drastic decline of the developed muscle force (Table I) Hypertonic infusions elicited dilator effects throughout prolonged infusion periods, but the dilator response was then usually maintained at a relatively steady level despite a gradual rise of osmo-

lality (Fig 7) A myogenic constrictor mechanism, as discussed above, could possibly explain this maintenance of flow (vascular tone) Tissue hyperosmolality may thus be importance for the exercise vasodilatation not only in early but also in later phases of continuous work at least if it is not too strenuous. Such a maintained dilator effect would be present provided the substances responsible for the work induced tissue hyperosmolality do not readily penetrate into the vascular smooth muscle cells as will be commented upon in General Discussion. In that chapter studies from other laboratories concerning vascular effects of hypertonic infusion will also be considered

CHAPTER III

CHANGES OF THE PRESSURE DROP CURVE AND OF RESISTANCE ALONG THE VASCULAR BED OF MUSCLE EVOKED BY EXERCISE AND "EXPERIMENTAL HYPEROSMOLALITY"

The results presented in the previous chapter showed that the patterns of vascular response to exercise and experimental hyperosmolality were quite similar with regard to the effects on the overall resistance function on the precapillary sphincters, and on the capacitance vessels. These findings thus supported the hypothesis that tissue hyperosmolality produced by muscle work is a mediator of exercise hyperemia. The present study was undertaken to obtain a more detailed picture of the resistance function during exercise and experimental hyper osmolality insofar that the evoked changes of resistance in delimited sections along the vascular tree were analysed. This was done by measuring blood pres sure at various points from artery to vein as well as total muscle blood flow The collected data permitted detailed evaluation of induced changes of the pressure drop curve in the vascular bed during exercise and during the so called "peak" and "steady state" dilator responses to hypertonic infusion. With the aid of these pressure curves, the resistance changes in very delimited parts of the vascular tree could be approximately revealed This analysis suggested that the peak dilator response rather than the steady state response to hypertonic infusion may be most relevant for the quantitative evaluation of the role of tissue hyper osmolality in exercise hyperemia (*cf* Fig 14 Chapter II)

METHODS

Experiments were performed on 26 cats of both sexes weighing 2 8—5 0 kg Observations were made during constant pressure perfusion on the acutely denervated vascular bed of the lower leg muscles. The anaesthesia and the main surgical procedures were the same as described in Chapter II. Venous outflow of blood from the studied region was recorded continuously by the technique described in that section. The arterial inflow to the muscles was diverted from the femoral to the popliteal artery through a short shunt circuit in which a "mixing chamber" was inserted to ensure thorough mixing during hypertonic infusion (Chapter II)

Arterial inflow pressure (AP) venous outflow pressure (VP) as well as pressure from a small intra-muscular arterial vessel (SAP) were measured in all experiments. In 9 of these, pressure was also monitored from a small venous vessel (SVP) AP and VP were measured close to the cannulated popliteal artery and vein from T-tubes The cannulas inserted into these vessels were found to

cause no measurable pressure drop at any of the flow rates encountered. The SAP and SVP were obtained using in principle the method described by Haddy *et al* (1954) implying that pressure in small vessels within the tissue is transmitted *via* collaterals to cannulated and proximally occluded vessels. For these measurements *the sural artery and vein on the lateral surface of the gastrocnemius* muscle were ligated and cannulated distal to the point of ligation with drawn-out flexible nylon tubings. Test experiments showed that an induced pressure change was transmitted with no measurable delay through these cannulas. By this cannulation technique SAP measurements were obtained, apparently largely at random, from fairly large down to small precapillary vessels (*cf* Results section) To obtain more regularly pressure measurements from the smallest precapillary vessels the arterial cannula was instead inserted in a small branch of the sural artery and advanced along the branch into the muscles. In some experiments two SAP measurements were obtained simultaneously by using both these cannulation techniques. AP and SAP were recorded with Statham P 23 AC transducers and VP and SVP with Statham P 23 DC transducers connected to a Grass Polygraph. Sometimes VP was recorded with an Elema pressure transducer on an Elema Electrocardiograph (Model 81) The pressure recording systems (baseline, calibration) were tested frequently during the course of the experiments against standard pressure (saline columns) AP was recorded as the mean pressure via electrical integration. During exercise SVP and VP were also recorded as mean pressures to eliminate phasic pressure changes induced by the muscle contractions. The tubings between the places of pressure measurement and the pressure transducers were filled with heparinized saline solution and flushed at intervals during the experiments. A free communication between the small intramuscular vessels and the vessels cannulated for SAP and SVP measurements was shown in several ways. Quick drainage of blood was obtained when the small pressure cannulas were disconnected from the pressure transducers further SAP and SVP rapidly changed when the arterial inflow or venous outflow tubings were occluded, a test performed at intervals for a few seconds throughout each experiment. A few experiments in which free communication was not present were discarded.

Muscle exercise (single twitches) was produced by electrical stimulation of the distal end of the cut sciatic nerve using a Grass stimulator model S 4K. The stimulation characteristics (5 V 0.5 msec, 0.25—4 imp/sec) were chosen so as to avoid any excitation of the sympathetic fibres (see Chapter II Methods) Hypertonic ($\approx$1500 mOsm/kg H_2O) solutions (usually glucose but sometimes sodium lactate) were infused into the arterial shunt at rates between 0.3 and 1 9 ml/min using a constant infusion apparatus.

Total vascular resistance (R_T) was calculated as (AP — VP)/flow Resistance proximal (R_P) and distal (R_D) to the points in the vascular tree from where small vessel pressures were derived could be defined with regard to the SAP as well as with regard to the SVP measurement. Thus R_P = (AP — SAP)/flow

or R_P = (AP — SVP)/flow R_D = (SAP — VP)/flow or R_D = (SVP — VP)/flow (SAP — SVP)/flow gave small vessel resistance.

RESULTS

In the experiments to be reported mean arterial pressure at the place of the popliteal artery (AP) averaged 106±1.7 SEM mm Hg and popliteal venous out flow pressure (VP) 6±0.2 SEM mm Hg in the resting control period before experimental intervention. Small artery pressure (SAP) in the control period varied between 88 and 13 mm Hg and small venous pressure (SVP) between 30 and 9 mm Hg. It should be pointed out that in the individual experiment control SAP almost invariably exceeded control SVP In order to standardize the experimental conditions and make possible a valid comparison between results obtained during exercise and during hypertonic infusion only those experiments were accepted in which resting control total vascular conductance (resistance) could be considered normal according to the criteria given in Chapter II A3 This implied that control vascular conductance varied between 0 053 and 0 117 (ml/min x 100 g)/mm Hg. Under these standardized conditions the variation in the magnitude of the small pressures in the control state could be regarded as a sign that pressure measurements were obtained from distinctly different points along the vascular tree. The SAP (88 — 13 mm Hg) could thus be considered derived from proximal down to the smallest precapillary vessels. In between each experimental intervention a sufficient time period elapsed to allow the small vessel pressures and the vascular resistances (R_T R_P R_D) to return approximately to their control values.

A. Exercise experiments

Fig. 15 shows representative original tracings of blood flow and of the various pressures during graded exercise. In response to the step-wise rate of somatomotor fibre stimulation blood flow increased from a control value of 6.3 ml/min x 100 g to 12.6 ml at 0.5 imp/sec, to 19 0 ml at 1 imp/sec, and to 62 ml/min x 100 g tissue at 4 imp/sec. AP was about 95 mm Hg in the control period and did not change much during the exercise whereas VP increased gradually from 6 up to 10 mm Hg. SAP was 56 mm Hg in the control period and it declined soon after the commencement of exercise to reach within about one minute, a steady state level of about 31 mm Hg at 0.5 imp/sec. At 1 imp/sec there was some further pressure drop to 29 mm Hg while at 4 imp/sec SAP again rose to 33 mm Hg. SVP increased from 14 mm Hg to about 18 20 and 28 mm Hg during the three exercise periods. Upon cessation of exercise, blood flow VP and SVP showed a gradual recovery to the control values, reached in about 15 min. SAP showed a slight further decline soon after work and then a slow return towards the control level. At the time when blood flow had returned to the control level, SAP was still some 15 mm Hg below the control value and it took another 15 min for complete recovery (note that no significant *abrupt*

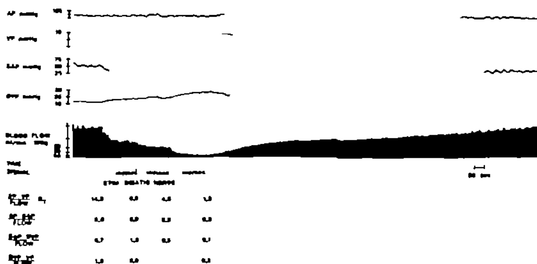


Fig. 15 Tracings of blood flow and of arterial inflow pressure (AP) venous outflow pressure (VP), small artery pressure (SAP), and small vein pressure (SVP) in skeletal muscle during graded exercise. Calculated values for total resistance (R_T) and for resistances in various segments of the vascular bed shown at bottom of the figure.

change in SAP SVP or VP occured in the immediate postcontraction period indicating that the single twitches *per se* did not contribute to the observed pressure changes during work)

The pressure in a small vessel at any moment is determined by the magnitude of AP and VP and by the relation between the vascular resistances distal (R_D) and proximal (R_P) to the point in the vascular tree from where the pressure is derived At constant AP and VP a relatively more pronounced decrease of distal resistance than of proximal resistance leads to a decrease of small vessel pressure whereas when the relative resistance changes are the opposite ones, small vessel pressure increases. R_D/R_P can be calculated for the points from which small artery as well as small vein pressures were measured. With regard to the SAP measurement in Fig. 15 R_D/R_P changed from a control value of 1.32 to 0.38 during exercise at 0.5 imp/sec, to 0.32 at 1/sec, and to 0.40 at 4/sec. The observed changes of SAP can be mainly ascribed to the changes of R_D/R_P, since AP and VP did not alter much. With regard to the point of SVP measurement, R_D/R_P increased progressively with increasing rate of somatomotor fibre stimulation Information about changes of total and segmental vascular resistances in absolute terms can be obtained from the concomitant observations of the pressures and blood flow These data for the described experiment are shown at the bottom of the record (Fig. 15) Special mention may be made to the fact that during light exercise (0.5/sec) the fall in R_T was accomplished mainly by a dilatation of the small vessels, see (SAP — SVP)/flow whereas during heavy exercise (4/sec) the additional fall in R_T to a great extent

also depended upon relaxation of the larger resistance vessels situated proximal to the point of the SAP measurement, see (AP — SAP)/flow Note further that, especially during heavy work the pressure drop increased markedly in the proximal arterial and the distal venous segments, while the pressure drop across the small vessels was considerably reduced. At the time after exercise when blood flow and R_T had returned to the control levels, i.e towards the end of the registration, SAP was still not restored This was due to a redistribution of vascular resistance insofar that proximal arterial resistance (AP — SAP)/flow was somewhat larger and small vessel resistance, (SAP — SVP)/flow correspondingly smaller than in the control period at rest.

A relative estimation of the level in the vascular tree at which small vessel pressure is measured in this type of experiment can be obtained by calculating the ratio of R_P/R_T at resting vascular tone in the control period. This permits a functional division of the vascular tree with regard to resistance. The ratio approaches zero when "small vessel pressure" is derived from the most proximal arterial vessel and approaches unity when it is derived from the most distal venous vessel A value of 0 80 to 0 85 may be taken to represent the capillary level, assuming a capillary hydrostatic pressure of 20 to 25 mm Hg at normal pressure head (see below)

In the experiment just described, R_P/R_T calculated for the SAP value in the control period was 0.43 Tables III and IV show representative data on the changes of pressure and resistance in response to graded exercise obtained in experiments where SAP was derived from a more proximal point (Table III) and a more distal point (Table IV) than in this experiment. Thus, R_P/R_T in the control period was 0.21 in the former experiment and 0 75 in the latter The data in these two tables,like those of the experiment shown in Fig 15 show that the main resistance decline during exercise occurs in the smaller precapillary vessels. It may be noted from Table III that, although R_T decreased during light (0.25 imp/sec) exercise, proximal arterial resistance in fact concomitantly increased above the control level by some 20 per cent. Such a resistance increase was a constant finding in experiments where the most proximal arterial segment was analysed. The resistance increase in this segment can possibly be ascribed to passive factors (see Comments) However as work intensity increased (0.5 1 0 and 4 imp/sec) this effect was overcome by a dilator influence of exercise. Note should also be taken in Table III of the very steep pressure drop across the proximal arterial segment and the concomitant small pressure drop across the vessels located distal to the point in the vascular tree from where SAP was derived.

From the experiment in Table IV a rough estimate of the changes of mean capillary pressure during exercise may be obtained, since SAP and SVP were apparantly derived from points relatively close to the capillary level. Assuming that mean hydrostatic capillary pressure (P) in this experiment roughly can be taken as the mean of SAP and SVP P in the control period would be 25 mm Hg, at 0 75 imp/sec 22 mm Hg and at 4 imp/sec 29 mm Hg. These figures

TABLE III

	AP mm Hg	SAP mm Hg	VP mm Hg	Blood flow ml/min x 100 g	(AP—SAP)/ Flow = R_P mm Hg / ml/min 100 g	(SAP—VP)/ Flow = R_D mm Hg / ml/min x 100 g	(AP—VP)/ Flow = R_T mm Hg / ml/min x 100 g
Control	95	77	8	6.6	2.7	10 5	13.2
Exercise 0.25/sec	93	60	8	9 9	3.3	5.3	8 6
Exercise 0.5/sec	97	45	10	21 0	2.5	1 7	4 1
Exercise 1/sec	92	29	11	29.2	2.2	0.6	2.8
Exercise 4/sec	87	24	13	60 4	1 0	0 2	1 2

TABLE III Arterial nflow pressure (AP) small artery pressure (SAP), enous outflow pressure (VP), blood flow and calculated total resistance (R_T) and resistance proximal and distal to the site f SAP measurement during graded exercise. SAP in this experiment derived from a relatively proximal point in the vascular tree. For detailed descripeion see text.

TABLE IV

	AP mm Hg	SAP mm Hg	SVP mm Hg	VP mm Hg	Blood flow ml/min x 100 g	(AP—SAP)/Flow mm Hg / ml/min 100 g	(SAP—SVP)/Flow mm Hg / ml/min x 100 g	(SVP—VP)/Flow mm Hg / ml/min x 100 g	(AP—VP)/Flow mm Hg / ml/min x 100 g
Control	115	33	16	6	10.0	8.2	1 7	1 0	10.9
Exercise 0 75/sec	110	23	21	8	15 4	5.7	0 1	0.8	6.6
Exercise 4/sec	100	29	28	10	37.7	1 9	0.0(3)	0.5	2.4

TABLE IV Data from similar experiment to that shown in Table III. SAP derived from a relatively distal point in the vascula tree. For detailed description see text.

suggest that P_c is not changed very much by exercise, the effects of the rise in SVP to a great extent being balanced by the fall in SAP It should be stressed therefore, that an observed change of SVP *per se* cannot, as sometimes is done be taken as an indicator of a corresponding change in P The data also suggest that resistance and hence the pressure head across the capillaries can indeed be small during exercise.

From an analysis of the changes of segmental resistances during the development of the exercise hyperemia in the initial phase of graded exercise the following conclusions could be drawn. During light exercise there was a gradual dilator response reaching a steady state level within 1—2 min and this response preferentially was confined to the small vessels. During heavy exercise there was a more rapid dilator response which initially also mainly involved the small vessels. As R_T approached its low level the more proximally situated arterial resistance vessels were also recruited in the dilator response. With regard to the *post-exercise period and especially after heavy and prolonged work, a regular* observation was that SAP remained low for a considerable time after that R_T and blood flow had been restored to the control levels. Thus, while R_T was restored within 10 — 25 min sometimes SAP did not return to the control level until 60 or more minutes after the cessation of the work. As mentioned above, maintenance of a low SAP value could be ascribed to a redistribution of vascular resistance, proximal arterial resistance being larger and small vessel resistance smaller than in the control period at rest. This redistribution of vascular resistance apparently implies that P in the post-exercise period is *lower* than in the control period before work and such a drop of P can aid in the reabsorption of the fluid that has accumulated in the extravascular space of muscle during exercise (*cf* Chapter IV)

Since in the different experiments SAP and SVP measurements were obtained from various levels in the vascular tree, the collected data permit a description of the changes in the pressure drop curve from artery to vein induced by exercise. The level at which small pressure was derived in the individual experiment could be defined by the control value for R_P/R_T observed in the muscle vascular bed at rest ("(R_P/R_T) control") In Fig. 16 the pressure drop from the popliteal artery to vein is plotted against the calculated values for (R_P/R_T) control. The values for (R_P/R_T) control which ranges from zero (popliteal artery) to 1 0 (popliteal vein) are plotted on a linear scale on the horizontal axis. The vascular bed is thus for the resting control state divided arbitrarily into "equi resistant units implying that for this state the pressure drop curve from popliteal artery (mean AP 105 mm Hg) to popliteal vein (mean VP 5 mm Hg) forms a straight line. For reasons discussed below mean hydrostatic capillary pressure in the control state was considered to be 22 mm Hg, on the average, and the pressure drop along the capillaries 6 mm Hg. By these values the capillary section can be delimited on the horizontal axis in the diagram of Fig. 16 and, hence, also the precapillary and postcapillary vascular sections. Implicit in the method of seg

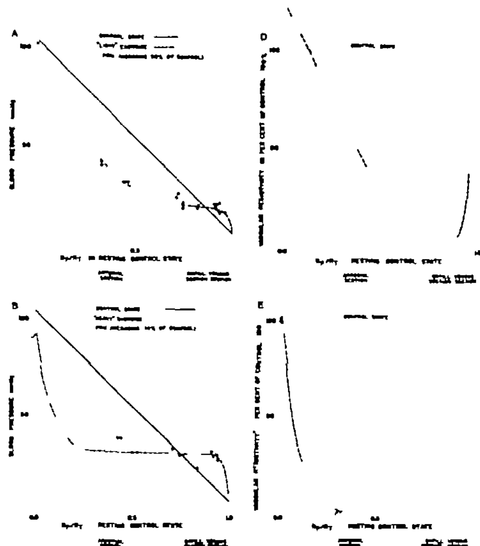


Fig. 16. Changes of the pressure drop curve from artery to vein and of vascular resistivity along the vascular tree during "light" (panels A and D) and "heavy" (panels B and E) exercise. Vascular tree functionally divided into equiresistant units. Closed circles SAP measurements, open circles SVP measurements. For further description see text. Panel C shows the pressure drop in the morphological sections of the vascular tree for resting control state (————); light exercise (O O) heavy exercise (● ●) the broken line shows conventional representation of the pressure drop curve during vasodilatation.

mental pressure recording is that small vessel pressure during experimental intervention is derived from the same site as in the control situation Thus, in Fig. 16, the individual observations of small artery and small vein pressures during exercise have been plotted against the given corresponding values for (R_P/R_T) control. As mentioned above, the induced small pressure changes varied with the decline in R_T reflecting work intensity Therefore the material has been classed and the plotted data refer to two levels of R_T change panel A to experiments in which R_T decreased by less than 60 % ("light exercise") and panel B to experiments in which R_T decreased by more than 80 % ("heavy exercise") of resting control resistance (it may be mentioned that the spread of data in the diagrams can partly be attributed to the variations of the induced change of R_T that still were present in each class of data) In these diagrams the values for VP during exercise refer to the mean value of all observations in each group of experiments. Since AP varied slightly between the individual experiments, all values for SAP and SVP have been corrected in the diagrams so as to be relevant for the mean AP of 105 mm Hg using the experimentally found R_P/R_T values in the recalculation. Test experiments, in which the influence of a mechanically produced change in AP on small vessel pressures was observed directly showed that the error introduced by such a recalculation was quite insignificant. The smooth curves in the diagrams show the approximate shapes of the pressure drop curve from artery to vein during light and heavy exercise. The curves are fitted by eye over the range of experimental observations and extrapolated further to the AP and VP values. It can be seen that exercise caused a marked shift of the pressure drop curve. Thus, the pressure drop in proximal arterial and distal venous vessels was greatly increased, wheras concomitantly that across the small vessels became reduced. These changes were more pronounced the heavier the exercise. The curves further indicate that mean hydrostatic capillary pressure (P) was largely unaltered (or even decreased) during light exercise but that it rose by some 8 mm Hg during heavy work. Note again that the effect on P of the elevated SVP to a significant extent was balanced by the concomitant drop of precapillary small vessel pressure. The pressure drop curve for those experiments in which the decline in R_T was greater than in panel A but less than in panel B "moderate work" is omitted due to limited space in the diagram As expected, this curve was situated between the curves in panels A and B and with regard to the capillary pressure it indicated that P was increased by some 3 — 4 mm Hg.

It should be pointed out that the vascular tree in panels A and B of Fig. 16 with regard to resistance has been considered from a functional point of view and that here it was divided into "equi-resistant" units. These data cannot be directly transformed so as to depict the pressure drop in the various consecutive morphological sections of the muscle vascular bed (arteries, arterioles, precapillary sphincters, capillaries, venules, and veins) since detailed concomitant morphological and functional studies are still lacking. No doubt the major pressure drop in

the resting vascular bed *occurs in the small precapillary vessels (especially in the arterioles)* The pressure drop curve (solid line) in panel C of Fig. 16, familiar from textbooks etc, may thus reflect the situation for a muscle vascular bed (feeding artery to draining vein) with normal tone Since this curve corresponds to the straight line control pressure curve in panels A and B (with the reservation that the vascular bed was denervated in the present study) it is possible to rearrange the data observed during exercise (smooth curves in panels A and B) so as to depict in approximate terms the pressure drop across the various morphological vascular sections (panel C, light exercise open circles, heavy exercise closed circles) Thus the major pressure drop during vasodilatation evoked by heavy exercise apparently occurs in small and medium-sized arteries instead of in the arterioles The broken line shows how the pressure drop curve during vasodilatation in principle has been visualized conventionally *(cf* Guyton 1966, Folkow and Neil 1971) The present report, in which a more systematic study was performed of the pressure drop in various segments along the vascular tree, strongly suggests that this conventional curve may not be correct at some critical *points. It should be pointed out in this connection that the pressure drop curve* seemed quite similar to that described for exercise when in an additional series of experiments, vasodilatation was produced in the resting skeletal muscle by close arterial infusion of papaverine or acetylcholine.

The pressure drop curves presented in Fig 16 enable the calculation, in approximate terms, of the changes in resistance in response to exercise in any delimited part of the vascular bed. Such treatment of the data would give a more refined analysis than the above described changes of gross segmental resistance. Although each curve in panels A and B gives information about the distribution of resistance to flow from artery to vein at the prevailing R_T levels, the evoked changes of resistance by exercise in the various vascular sections cannot be evaluated unless the changes in blood flow are taken into consideration The blood flow values observed in the control states and during light and heavy exercise were *recalculated to be valid for the perfusion pressures* (AP — VP) *in panels* A *and* B (it should be noted that the error introduced by such a recalculation is small when the variations in AP is limited *cf* Chapter II Results Section A 3) It was found that, from a mean resting control blood flow of 7 8 ml/min x 100 g, flow on average increased by a factor of 2 0 during light exercise. During heavy work it increased by a factor of 6 8 from a mean resting value of 7 1 ml/min x 100 g. It is clear that for each given state, each section of the vascular bed is traversed by the same volume flow (apart from smaller differences due to net transcapillary fluid exchange, see below) Resistance at any point, denoted "vascular resistivity may therefore be obtained from the slope of the curves relating pressure (P) to (R_P/R_T) control (Fig 16 A and B) and from the blood flow according to the equation

$$\text{"resistivity"} = -\frac{dP}{d[(R_P/R_T)\ \text{control}]} \qquad \frac{1}{\text{flow}}$$

The changes of vascular resistivity induced along the vascular bed during light and heavy exercise are shown in panels D and E of Fig. 16 with the exception of the very distal part of the venous segment which was omitted due to the difficulty in extrapolating the pressure curves (Panels A and B) down to VP. Since, for the control state, the vascular tree was divided into "equi-resistant" units (straight line in Fig. 16 A and B) control resistivity is constant throughout the vascular bed and has been set to 100 per cent. The resistivity during exercise is expressed in per cent of control. Therefore exercise values below 100 per cent denote a decrease of resistivity and values above 100 per cent an increase of resistivity compared to the control state. The diagrams give a graphical description of the changes of resistivity along the entire vascular bed and the integral of the curves (areas under the curves) depicts the vascular resistance distribution.

It can be seen that light as well heavy exercise induced a decrease of vascular resistivity in all but the most proximal parts of the vascular tree. During a relatively small dilator response, as produced by light exercise, the most pronounced changes of resistivity occurred in the microvessels, whereas during heavy exercise marked resistivity changes also occurred in the somewhat larger vessels, especially on the precapillary side. These changes can be ascribed mainly to relaxation of the vascular smooth muscle, the effect in the capillary section being related to an opening up of "precapillary sphincters" greatly increasing the number of parallel-coupled capillary channels. It appears that rheological effects, e.g. decreased blood viscosity, can contribute to the resistivity decline (see Comments). In the somewhat larger venous vessels ("capacitance vessels") the decrease of resistivity may be caused mainly by passive distension due to the increased transmural pressure (*cf.* Chapter II, Results section C). The increased resistivity in the most proximal arterial vessels during light exercise is in agreement with the changes of segmental resistance described above (Table III) and the present analysis suggests that such an effect may be present also during heavy exercise in the vessels quite close to the conduit artery. The drastic decrease of resistivity in the small vessels during heavy exercise might at first sight appear surprising but seems plausible in view of the fact that R_T decreased by more than 80 % and that resistivity decreased much less in other sections of the vascular bed.

B. Hypertonic infusion experiments

These experiments were performed on preparations in which total vascular resistance (R_T) in the control period before infusion was of about the same magnitude as in the above described exercise experiments and in which the hypertonic infusion rates were adjusted so as to elicit graded decreases of R_T over approximately the same range as encountered in the exercise experiments. The experimental hyperosmolality was produced by i.a. infusion of glucose or sodium lactate solutions which were found to elicit similar effects.

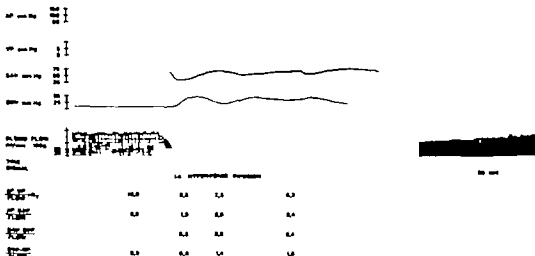


Fig 17 Tracings of blood flow and of arterial inflow pressure (AP), venous outflow pressure (VP) small artery pressure (SAP), and small vein pressure (SVP) in resting muscle during hypertonic infusion. Calculated values for total resistance (R_T) and for resistances in various segments of the vascular bed shown at bottom of the figure.

Fig 17 shows representative original tracings of blood flow and of the various vascular pressures during hypertonic glucose infusion (signal) At the bottom of the record calculated values for R_T and the segmental resistances are given. It can be seen that the infusion elicited a typical pattern of blood flow increase (*cf* Chapter II Fig 5) *i.e* an initial peak response followed by some return of flow (steady state response) Thus, flow increased from a control value of 5.6 ml to 29.3 ml at the peak and to 12 — 15 ml/min x 100 g tissue in the steady state phase. AP was 106 mm Hg in the control period and remained at about this level throughout the experiment except for a slight transient fall early during infusion. VP increased by a few mm Hg from a control value of 6 mm Hg. SAP was 61 mm Hg in the control period corresponding to an R_P/R_T value of 0 45 During the hypertonic infusion there was an initial slight, transient increase of SAP possibly an effect of the fluid infusion *per se* and of the evoked transcapillary fluid absorption. SAP then showed a rapid fall and reached a low level of 35 mm Hg by the time the peak flow response was almost but not fully developed. This decrease mainly can be ascribed to a relatively more pronounced dilatation of the vessels located distal to the point of measurement than in the proximal vessels and to a slight extent to the concomitant fall of AP It should be noted that at the time blood flow was approaching its peak value, SAP started to increase again and rose by about 5 mm Hg during the short phase when blood flow was maintained constant at this high level This effect which is more clearly shown in the next figure, can be ascribed to a progressive resistance decrease in the proximal vessels soon followed by the commencement of a resistance increase in the distal vessels SAP then showed a more pronounced increase concomitant

to the gradual return of flow towards the steady state level and reached a value of 69 mm Hg in the early steady state phase after which it stabilized at about the control value. This shows that the return of flow from peak to steady state is due to a relatively more pronounced partial recovery of resistance in the distal than in the proximal segment which is also clear from the figures for absolute resistances. SVP increased from a control value of 19 mm Hg to a maximal value of 34 mm Hg and it stabilized after some oscillation at a value of about 30 mm Hg during the infusion. The SVP increase was related to a relatively more pronounced resistance decline in the vessels proximal than distal to this site of pressure measurement. Upon cessation of the infusion blood flow and the pressures gradually returned to the control values. Thus the redistribution of vascular resistance observed after exercise was not present. The small initial changes of SAP and SVP at the end of the infusion may be related to osmotic transcapillary fluid flux into the tissue occurring when the infusion ended Summarizing, Fig. 17 shows that the decreases of R_T in the peak and the steady state phases can be ascribed to dilator effects in all the three vascular segments. During the peak response the relative change in resistance in most pronounced in the vessels located distal to the SAP measurement although the proximal segment contributes to an almost equal resistance decline in absolute terms. Recovery of vascular tone from peak to steady state response is mainly due to constriction of the small vessels.

Fig 18 shows changes of blood flow and SAP during hypertonic infusion in an experiment where the SAP measurement was derived from a more distal point ((R_P/R_T) control = 0.72) This tracing may serve to illustrate the not infrequently observed finding that after a transient fall of SAP during the development of the flow increase (in this case from 40 to 27 mm Hg), there was a marked increase of SAP at a time clearly preceding that at which blood flow started to return to the steady state level (in this case from 27 to 48 mm Hg) When the steady state flow was established, SAP significantly exceeded its control value. This was the usual finding in experiments in which SAP was obtained from the smallest precapillary vessels. The extent to which R_T R_D, and R_P changed during various phases of the hypertonic infusion is shown by the figures at bottom of the record. Since AP and VP were not much changed, the alterations of SAP can mainly be ascribed to the changes of R_D/R_P The values for R_D and R_P and for R_D/R_P shows that during the early dilator response, vascular resistance declines in both the proximal and distal segments, and that the relative change is greatest in the distal one. During the maximal flow increase there is a continuing resistance decline in the proximal segment whereas distal resistance starts to increase again, both these effects contributing to the increased R_D/R_P value. Return of flow to the steady state mainly depends on increased distal resistance. Thus the rise of transmural pressure in the small vessels, which is probably reinforced by a concomitant decrease of tissue pressure due to the osmotic transcapillary fluid absorption, is a phenomenon which starts to develop early during

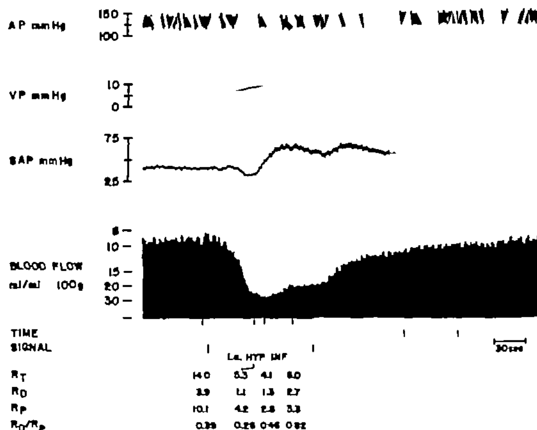
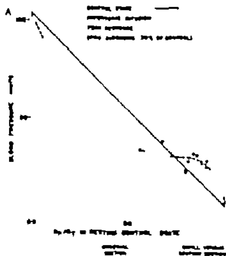
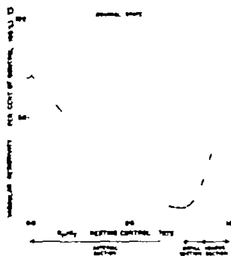


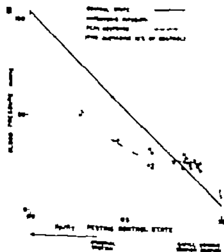
Fig 18 Tracings of blood flow and of arterial inflow pressure (AP), venous outflow pressure (VP), and small artery pressure (SAP) in resting muscle during hypertonic infusion. Calculated values for total resistance (R_T), for resistance proximal (R_P) and distal (R_D) to the point in the vascular tree of the SAP measurement, and for the ratio R_D/R_P shown at bottom of the figure. Note the marked SAP increase commencing during the peak flow response to hypertonic infusion which tentatively can explain the later partial recovery of vascular tone (steady state flow response) by the elicitation of a secondary myogenic constrictor effect.

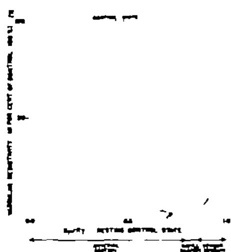
Fig 19 Changes of the pressure drop curve from artery to vein and of vascular "resistivity" along the vascular tree during hypertonic infusion. Panels A and D "peak response" data for "moderate" decrease of R_T. Panels B and E: "peak response" data for "large" decrease of R_T. Panels C and F: "steady state response". Vascular tree functionally divided into equi-resistant units. Closed circles SAP measurements, open circles SVP measurements. For further description see text.

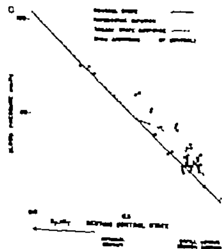
hypertonic infusion. As will be discussed below this transmural pressure rise might elicit a myogenic constrictor response in these vessels counteracting the dilator action of hyperosmolality and, hence, at least partly be responsible for the return of blood flow from the peak to the steady state level and, further for the maintenance of flow (vascular tone) in the later phase. It should be stressed that such a transmural pressure increase was not observed during exercise.

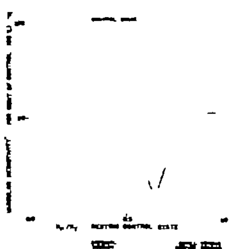












As during the exercise experiments described above small vessel pressure measurements (SAP as well as SVP) were obtained from various points along the vascular bed from artery to vein as shown by the different values for (R_P/R_T) control. The collected data during the hypertonic infusion experiments are shown in Fig. 19 and are presented in a similar way to that used for exercise (Fig. 16), i.e the pressure drop along the vascular bed has been plotted against (R_P/R_T) control (panels A, B and C) Curves for evoked changes of "vascular resistivity" during hypertonic infusion have also been derived (panels D E and F). As in the exercise diagrams (Fig. 16), resistivity changes in the most distal venous vessels have not been calculated. Panels A and B represent the pressure data during peak response, panel A for experiments in which R_T decreased by less than 75 % (mean decrease 61 %) and panel B for experiments in which R_T decreased by more than 75 % (mean decrease 81 %) These changes of R_T are roughly comparable to those evoked in the exercise experiments shown in panel A and B of Fig. 16 Panel C shows the corresponding data during the early steady state response, i e in a phase about 1 to 3 min after the start of the hypertonic infusion. Since in this phase of the dilator response to hypertonic infusion no significant systematic difference with regard to small vessel pressures was found when the R_T decline was large or when it was small the plotted data refer to all the observations (mean decrease of R_T being 59 %).

It can be seen from Fig. 19 that during the peak response (panel A and B) the general characteristics of the pressure drop curves along the vascular bed were similar to those observed during light and heavy exercise *(cf* Fig. 16) However during the more pronounced decrease of R_T (Panel B) the pressure drop in the proximal arterial vessels was smaller and in the more distal arterial vessels greater than during heavy exercise. Further it can be seen that pressure in the smallest precapillary vessels tended to be somewhat higher during the infusion experiments than during exercise, especially at the smaller R_T decline. The curves for "resistivity" changes during the peak response roughly resemble those derived during exercise, although some differences seem apparent. Thus, hypertonicity seems to affect the more proximal arterial vessels to a greater extent and the more distal arterial vessels to a somewhat lesser extent than exercise. With regard to the steady state response during hypertonic infusion, the pressure drop curve from artery to vein largely followed the control curve, and was thus clearly different from those depicted for the peak response and for exercise. It can be inferred from the shape of this pressure drop curve that, although R_T on the average was significantly less than in the control state, the distribution of resistance along the vascular bed during the steady state response was not much different from that in the control state. Consequently the "resistivity" curve for the steady response showed a virtually constant change of resistivity throughout the vascular bed with the exception for the smallest vessels where it decreased more than in the larger vessels. The difference in the shape of the resistivity curves between the peak and the steady state response could be ascribed mainly to the

preferential constriction of the smaller vessels (apparently including the "pre-capillary sphincters") that occurs during the latter response (*cf* Figs. 17 and 18) This was also evident when calculating separetely the resistivity changes for the steady state response observations in the infusion experiments from which the peak response data in panels A and B, respectively were derived.

COMMENTS

In the present study the changes of pressure and of resistance along the skeletal muscle vascular bed in response to exercise and hypertonic infusion were defined in greater detail than in previous literature (*cf* Haddy *et al* 1954 Folkow *et al* 1971 Gazitúa *et al.* 1971). The experimental approach used is based in principle on the well-known method of Haddy *et al* (1954) for small vessel pressure mea surements and segmental resistance calculations. The processing of data in the present study seems, however to provide much more detailed information. The validity of the resistance calculations based on measurement of small vessel pres sures has been discussed at length by others (*e g* Folkow *et al* 1971) Only a few comments will therefore be made.

In most studies of segmental vascular resistance constant flow perfusion has been used which has the advantage that resistance effects are reflected directly by the observed pressure changes. An obvious drawback with constant flow per fusion is, however that unphysiologic pressure changes are induced during va sodilatation, for instance, AP often falls to such low levels that secondary pas sive alterations in vessel diameter to an abnormal extent might interfere with the active vascular response. The more physiological approach with constant pressure perfusion was used in the present study

The same volume flow of blood should pass each consecutive section of the vascular bed at a given moment and it is assumed with this method that there is a uniform distribution of flow in the various sections. This assumption seems reasonable for the larger pre- and postcapillary vessels but, as pointed out by Folkow *et al.* (1971), blood flow in the smaller vessels may show considerable random variation. Therefore results from a single experiment may not neces sarily be conclusive but, as stressed by these authors, uniform results from a large series of experiments, as in the present study should provide relevant informa tion (Mention should be made here that as a result of the net transcapillary fluid fluxes which are evoked by exercise and by hypertonic infusion, the volume flow in pre- and postcapillary vessels was in all probability somewhat different in the present study (*cf* Chapter II). The consequences of this for the results presented will be considered below) Another assumption is that small vessel pressure is transmitted through the same collateral vessel in the control situation and during experimental intervention this appears likely but cannot be proved. Again, col lecting data from many experiments would seem to minimize the error if the above should not always be true. Another question of importance, especially when constant pressure perfusion is used, is if kinetic energy forms a significant and

variable part of the total energy which could affect the pressure measurements and give rise to erroneous conclusions. Calculations of this factor for the various flow rates encountered indicated that such an error must be insignificant, which is further supported by the critical analysis of the problem presented for instance by Burton (1966)

Attempts were made to obtain small vessel pressures from widely different points in the vascular tree by cannulation of various vessels. The large range of small vessel pressures in the control state indicates that the pressure were monitored from arterial and venous vessels of quite varying bores although not from the largest ones, perhaps since these have few collaterals. To some extent the variations of small pressure in the control state would seem caused by resistance variations Such an influence was minimized, however by accepting only those experiments in which control R_T could be considered "normal"

SAP values, say below 35 mm Hg, may be considered representative for quite small precapillary vessels, and the control observations below this pressure level averaged 26 mm Hg (11 animals mean AP 104 mm Hg and mean VP 6 mm Hg) SVP averaged 20 mm Hg (9 animals mean AP 109 mm Hg and mean VP 6 mm Hg) Control mean capillary pressure ought to be somewhere in between these values and probably closest to the SVP the figure of 22 mm Hg for P used in the diagrams (Figs. 16 and 19 above) thus seems reasonable and is in rough agreement with data given in the literature (*e g* Landis and Pappenheimer 1963 Smaje *et al.* 1970) although there are reports suggesting that P may be as low as about 15 mm Hg (*e g* Diana 1970) The figure of 6 mm Hg used for the pressure drop across the capillary is in accordance with findings on rat cremaster muscle (Smaje *et al* 1970) It should be pointed out here that due to possible variation of blood flow in the small vessels, observations of an especially low SAP or an especially high SVP do not necessarily provide information about mean P for *all* capillaries in the preparation.

The collected data on blood pressures and flow provided rough information about changes of the pressure drop curve and of resistance alterations in delimited parts of the vascular tree induced by exercise or hypertonic infusion (Figs. 16 and 19) It is evident, as can be expected, that the major resistance decline occurred in the small vessels. The surprisingly large decline even in the capillaries deserve comment (*it may be noted that should resting control P have been overestimated, say by some 5 mm Hg* the resistivity decline in the capillary section during heavy exercise would still be pronounced as suggested by Fig. 16) In the denervated vascular bed of the cat lower leg muscles the capillary filtration coefficient (CFC) reflecting the number of patent capillaries, increases by some 200 — 300 per cent during heavy exercise (*e g* Kjellmer 1964 *a* Lundvall *et al* 1967 Chapter II and IV this volume) Assuming the mean diameter and mean length of the capillaries opened up during exercise to be similar to those in the control state, these changes in CFC would correspond to a resistance decrease in the capillary section down to about $^1/_4$ of the control values. Some other fac

tors might contribute to the observed resistance decline for instance a possible viscosity decrease as flow velocity increases. Alternative explanations could be a possible distension of the capillaries, opening of arterio-venous shunt vessels etc, but there is experimental evidence that this does not occur in skeletal muscles (for ref see Mellander and Johansson 1968)

Somewhat puzzling is the quite regular observations that, especially during light exercise, there seemed to be some resistance increase in the most proximally situated arterial vessels (e g Table III) There is at present no explanation for this phenomenon even if it is likely that it is caused by passive factors. For instance, "nipping" of these vessels caused by muscle contraction (*cf* Gray *et al* 1967) or a passive elastic recoil due to the fall of transmural pressure could possibly have contributed to the effect.

A reason for undertaking this series of experiment was to get some indication of which of the resistance responses during hypertonic infusion, the peak or the steady state, should be considered in the quantitative evaluation of the role of hyperosmolality in exercise hyperemia. The one that showed a pattern of resistance response within the various parts of the vascular tree best resembling that evoked by exercise would seem of greatest relevance, especially if a deviating pattern could be regarded as caused by an "experimental artefact" (see below) It is clear from the present results (e g Figs. 16 and 19) that the peak effect was roughly similar to that during exercise whereas the steady state effect differed markedly It should be pointed out that as a result of transcapillary fluid transfer into the muscle during exercise and into the blood stream during hypertonic infusion, precapillary blood flow must have been underestimated during work but overestimated in the infusion experiments (Chapter II A 1 and A 2). Since this error should have been of greatest significance during the peak response to hypertonic infusion the main consequence would be that the resistivity changes during exercise and during the peak response in fact are more alike than indicated by the presented data. The deviating resistance pattern during the steady state response was caused by a partial but clear-cut recovery of vascular tone, preferentially in smaller vessels, upon transition from the initial peak response. A tentative explanation for this phenomenon is that a myogenic constrictor effect develops in response to raised transmural pressure in the small vessels. An increase of intraluminal pressure could thus be observed shortly before the partial recovery of tone and was marked and maintained during the steady state hyperemic phase (e g Fig. 18) The osmotic fluid absorption of extravascular fluid during hypertonic infusion would, in addition tend to decrease interstitial pressure thereby further raising transmural pressure, and such a decrease of interstitial pressure can apparently be quite significant in response to even small fluid shifts (Guyton 1965) In fact, the addition of fluid at the capillary level may also partly be responsible for the upstream intraluminal pressure increase. A stimulus for a myogenic constrictor response thus seems present during hypertonic infusion. That the small precapillary vessels, at least the "sphincters"

constrict upon increased transmural pressure during hypertonic infusion is shown in Chapter V To the extent that a high transmural pressure and a consequent myogenic constriction during hypertonic infusion is caused by extravascular fluid absorption *per se* and by a secondary decrease of tissue pressure it can be considered an "experimental artefact' inherent in the infusion experiments, not present when hyperosmolality primarily develops in the extravascular space as during exercise. Since a myogenic response during hypertonic infusion will be elicited with some delay after the stimulus develops it should not be prominent du ring the peak response. Therefore this dilator phase during hypertonic infusion may be more relevant than the steady state response when evaluating the role of hyperosmolality in exercise hyperemia (*cf* Fig. 14 Chapter II)

CHAPTER IV

MECHANISMS INVOLVED IN TRANSCAPILLARY FLUID MOVEMENTS DURING AND AFTER EXERCISE

It is well-known that during exercise there is a transcapillary fluid movement from the intravascular space into the extravascular compartment of the active muscles. Under certain circumstances, this process will be of great importance for general cardiovascular dynamics. Thus it has recently been shown that heavy exercise with large muscle groups can lead to a fluid accumulation in the active muscles of more than 1 000 ml in the human being and further that compensatory mechanisms are put into play so as to keep the consequent plasma volume reduction within tolerable limits (Lundvall *et al* 1970 1972). The mechanism behind the transcapillary fluid flux into exercising muscles was studied by Kjellmer (1964*a*) who arrived at the conclusion that it was solely due to filtration caused by increased capillary hydrostatic pressure. Our finding that exercise leads to considerable muscle tissue hyperosmolality (Mellander *et al* 1967 Chapter II this volume) strongly indicated that an osmotic process was also involved. The aim of the present study was to investigate the relative importance of these two mechanisms for the fluid transfer into exercising muscle. An attempt was also made to reveal the factors responsible for fluid return into the circulatory system after the cessation of work.

METHODS

41 cats (2.7—5 1 kg) of both sexes, anaesthetized as described in Chapter II were used in this study

The experiments were performed on the lower leg muscles which were enclosed in a water filled plethysmograph (38 C) permitting continuous recording of tissue volume. For surgical and technical details see Chapter II Arterial inflow pressure and regional venous outflow of blood were recorded continuously as described (Chapter II) Most of the experiments were performed under constant pressure perfusion In some of these, the arterial inflow was diverted from the ipsilateral femoral to the popliteal artery through a shunt circuit to permit intra-arterial infusions proximal to an inserted "mixing chamber" (Chapter II). In some experiments blood flow was controlled by the use of a perfusion pump (Harvard) on the arterial side.

Muscle exercise was produced by electrical stimulation (5V 0.5 msec, 0 25—4 imp/sec) of the distal end of the severed sciatic nerve using a Grass stimulator Observations were made during graded "short-term" (<5 min) and "prolonged" (≈15 min) exercise.

Some observations of the transcapillary fluid flux in resting muscle induced by hypertonic (≈1500 mOsm/kg) glucose infusion (0.1—1.0 ml/min) were also made. Infusion of papaverine hydrochloride (>0.6 mg/min) was sometimes given to produce a maximal dilatation of the vascular bed.

Blood samples (<1.5 ml) were taken for plasma osmolality determinations from a T-tube in the venous outflow tubing close to the popliteal vein and from a catheter in the right subclavian artery (exercise experiments) or from a T-tube in the arterial shunt distal to the mixing chamber (hypertonic infusion experiments) after discarding the "dead space" fluid volumes. To permit determination of the arterio-venous osmolar difference across the muscle vascular bed, arterial and venous blood samples were withdrawn simultaneously in the exercise experiments whereas in the hypertonic infusion experiments the sampling had to be separated slightly in time, the arterial sample being taken immediately after the venous sample. The osmolality measurements and compensation for the sampled blood volumes were performed as described in Chapter II.

Tissue volume was continuously recorded on a kymograph by the use of an air filled piston recorder connected to the plethysmograph. For the present purpose this mechanical recorder system showed greater sensitivity and stability than currently available electronic units. The sensitivity of the volume recording system could be varied so that a volume shift of 0.1 ml (corresponding, on the average, to about 0.15 per cent of the studied tissue volume) gave a deflection in the recording of 3—9 mm. By adjusting the height of the orifice of the venous outflow tubing from the muscle region, venous outflow pressure could be controlled. In the beginning of the experiments, venous outflow pressure was adjusted so that tissue volume was constant (isovolumetric state). Venous pressure was maintained at this level throughout the experiments except when otherwise stated or when the capillary filtration coefficient (CFC) was determined (see below).

Changes of tissue volume can depend on alterations in the regional blood content (capacitance responses) and/or on alterations in the extravascular fluid volume, the latter caused by net transcapillary fluid movement or lymph drainage. In the present study lymph drainage was prevented by ligation and cautherization of the regional lymph vessels. A large number of previous studies with the present technique have shown that under these circumstances the effects on tissue volume caused by transcapillary fluid fluxes and by changes of regional blood volume can be separated (for ref. see Mellander and Johansson 1968). In most cases this is quite easy since the time courses of the two phenomena are very different. Thus capacitance responses usually develop rapidly and are co-ordinated in time with the responses of the resistance vessels. It follows that net transcapillary fluid movements can be adequately determined as soon as a steady state vascular response is reached.

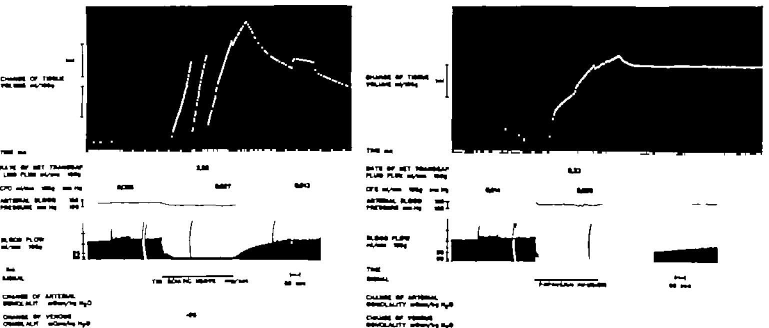
Data on the transcapillary hydrodynamic conductivity and changes in functional capillary surface area in the studied vascular bed were derived from de-

termination of the CFC, performed as described in Chapter II For the CFC calculations, it was assumed that 80 % of the venous pressure rise was transmitted to the capillary level during constant pressure perfusion (*cf* Cobbold *et al* 1963) and 100 % when flow was controlled by the perfusion pump (*cf* Kjellmer 1964 *a*). Muscle tissue weight was determined after dissection (see Chapter II).

RESULTS

The experiments illustrated in Fig. 20 were designed so as to permit an approximate quantitative evaluation of the net transcapillary fluid flux during exercise on the one hand caused by increased hydrostatic capillary pressure (filtration) and of the other caused by osmosis. Panel A shows representative changes of tissue volume and of blood flow in the lower leg muscles during relatively short-term heavy exercise (4 imp/sec) The capillary filtration coefficient (CFC) and arterial and venous osmolality was determined before and during the work. Blood flow was 7.2 ml/min x 100 g tissue in the resting control period and it increased rapidly at the commencement of exercise reaching a maximal and steady state level of 50.0 ml/min x 100 g after about 50 sec. Tissue volume, which was approximately constant in the control period (isovolumetric state), showed a most pronounced increase during exercise (volume recorder lowered twice during the period of work) A small fraction of the volume increase can be ascribed to an augmentation of regional blood volume (capacitance response mainly passive, *cf* Chapter II) an effect almost fully established at the time blood flow reached its maximal level (see Methods) The large tissue volume increase occurring during the phase of the steady hyperemia is therefore almost entirely due to a net transcapillary fluid movement from the intravascular to the extravascular compartment and it amounted in total to about 7 90 ml/100 g tissue in the 7 min work period (correction made for the effect of the CFC determination) The rate of transcapillary fluid flux was most rapid some two to three min after the commencement of exercise and then amounted to 2.52 ml/min x 100 g after which it tended to decline somewhat. It can be seen that exercise led to an increase of CFC from a control value of 0 008 to 0 027 ml/min x 100 g x mm Hg. At the time of maximal rate of transcapillary fluid movement, venous osmolality had increased by 26 mOsm/kg H_2O indicating that a considerable osmolar gradient from tissue to blood was caused by the work since concomitantly arterial osmolality increased by only 1 mOsm/kg H_2O Upon cessation of the exercise, blood flow tissue volume and CFC decreased Blood flow returned to the control level within about 9 min but a large fraction of the accumulated fluid was still present in the muscles.

The extent to which an increase of capillary hydrostatic pressure, by filtration, contributed to the transcapillary fluid flux in the described exercise experiment was approximately assessed from the experiment shown in panel B performed on the same animal The figure shows the tissue volume changes evoked



F g 20 Net transcapillary fluid transfer to skeletal muscl induced by exercise (panel A) and by i.a. papaverine i fusion (panel B), both caus ing maximal resistance essel dilatation. Fluid flux represented by the tissue volume increase (fter correction for nitial capacitance response). The pronounced fluid transfer during exercise can partly be ascribed to fluid filtration the extent of which is revealed by the infusion experiment; it can mainly be attributed to osmosis caused by work-induced tissue hyperosmolality

by close i.a. infusion of papaverine administered in a dose so as to elicit a blood flow increase of similar magnitude as in the exercise experiment, i.e. a virtually maximal resistance vessel dilatation. It can be seen that papaverine caused quite a marked initial volume increase (capacitance response) concomitant to the development of the hyperemia after which tissue volume increased at a much slower rate (net transcapillary fluid movement) The large capacitance response to papaverine (apparently greater than that evoked by exercise, panel A) indicates that, besides a passive distension of the capacitance vessels, this drug, like acetylcholine, also evokes active dilatation of these vessels (cf Chapter II) The transcapillary fluid movement amounted in total to about 1 50 ml/100 g tissue during the 6 min infusion period (correction made for the effect of the CFC determination) and the maximal rate of fluid flux was 0.33 ml/min x 100 g tissue. This transcapillary fluid flux must be ascribed to filtration caused by an increase of capillary hydrostatic pressure as a consequence of a decrease of the pre-/postcapillary resistance ratio, since, as shown by the figures for arterial and venous osmolality papaverine apparently caused no transcapillary osmolar gradient. The rate of net fluid filtration is determined by the magnitude of the capillary hydrostatic pressure rise and by the prevailing capillary hydrodynamic conductivity the latter reflected by the CFC, which was found to be 0 026 ml/min x 100 g x mm Hg during the papaverine infusion

As described in Chapter III exercise and papaverine seem to evoke roughly similar changes of capillary hydrostatic pressure (and hence of the pre-/ post capillary resistance ratio) in the muscle vascular bed at comparable decreases of total regional vascular resistance, despite the seemingly greater influence of papaverine on the capacitance vessels. Apparently the contribution to postcapillary resistance of the main capacitance vessels is small. The rise of capillary hydrostatic pressure in the present exercise and papaverine experiments should therefore be approximately equal since also the changes of the arterial blood pressure were similar in the two experiments. It follows that the net fluid filtration in the two experiments must have been about equally large, since CFC was of the same order of magnitude. Therefore, during the phase of maximal transcapillary fluid movement caused by exercise (2.52 ml/min x 100 g), less than 15 per cent of the fluid flux can be ascribed to fluid filtration (0.33 ml/min x 100 g). The remaining larger part of the fluid transfer is obviously due to an osmotic process caused by the transcapillary osmotic gradient developed in the exercising muscles as a consequence of the regional tissue hyperosmolality

The rise of mean capillary hydrostatic pressure occurring in the papaverine experiment can be calculated approximately by dividing the rate of fluid flux (0.33 ml/min x 100 g) by CFC (0 026 ml/min x 100 g x mm Hg) which gives a figure of 13 mm Hg from the foregoing discussion it follows that mean hydrostatic capillary pressure should have risen by the same amount in the exercise experiment. A similar deduction for the exercise experiment (maximal fluid movement divided by CFC) gives a figure of 93 mm Hg (2.52/0.027) The "ef

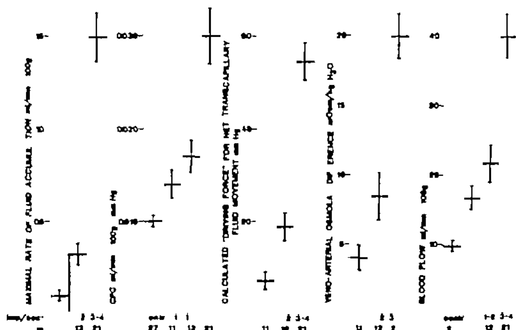


Fig 21 Diagram showing mean values ± SEM of the concomitantly determined maximal rate of net transcapillary fluid flux into exercising muscle, transcapillary filtration coefficient (CFC), transcapillary driving force for net fluid transfer (fluid flux/CFC), veno-arterial osmolar difference, and blood flow Data classed with regard to rate of somato-motor fibre stimulation (work intensity"). denotes number of observations in each class. CFC and blood flow in the resting control state also shown.

fective transcapillary osmotic force" induced by muscle work thus amounted to about 80 (93—13) mm Hg

At all frequencies of somato-motor fibre stimulation, reflecting work intensity the maximal rate of net transcapillary fluid movement from blood to exercising muscles was developed in the time period 2 to 4 min after the commencement of exercise. As will be shown, this can mainly be explained by the fact that the veno-arterial (transcapillary) osmolar difference and the hyperemia had then reached maximal levels. The hydrostatic capillary pressure rise should also have attained its maximum at this time.

Fig 21 shows mean data ± SEM from all exercise experiments (n = 44) performed under constant pressure perfusion for the following parameters maximal rate of fluid movement, CFC, calculated driving force for net transcapillary fluid movement, veno-arterial osmolar difference and blood flow The data refer to the time of maximal transcapillary fluid flux and are classed with regard to the somato-motor fibre stimulation frequency where rates <1 imp/sec may be considered as "light" exercise, 1—2 imp/sec as "moderate" and 3—4 imp/sec as "heavy" exercise. It can be seen that all the depicted parameters were

related to the stimulation frequency For the three stimulation ranges, the average rates of fluid movement were 0 10, 0.32 and 1 49 ml/min x 100 g tissue, respectively and the corresponding values for transcapillary driving force 7 19 and 50 mm Hg. The clear-cut veno-arterial osmolar differences suggest that an osmotic flux may be present at each work intensity The data reported in Chapter III indicated that capillary hydrostatic pressure on the average was largely unchanged during light exercise and rose by some 4 mm Hg during moderate, and by some 8 mm Hg during heavy work The latter figure is in accordance with a calculated average increase of capillary hydrostatic pressure of 9 mm Hg observed during maximal vasodilatation induced in the present study by i.a. infusion of papaverine (n = 6) The values for total driving force minus the mentioned figures for capillary hydrostatic pressure rise during exercise gives transcapillary osmotic forces for fluid flux of 7 15 and 42 mm Hg. More than 75 per cent of the fluid movement thus seems caused by osmosis at any one of the work intensities. Further comments on the data in Fig. 21 will be made later on.

The magnitude of the osmotic fluid movement from blood to the exercising muscles appears to depend on several factors. The fluid flux should be directly related to the established transcapillary osmolar gradient provided substances with similar osmotic reflection coefficients with regard to the capillary barrier are responsible for the interstitial hyperosmolality at different work intensities (for discussion of reflection coefficient, see Landis and Pappenheimer 1963) The transcapillary osmolar gradient and thus the fluid movement may in turn, be related to the magnitude of the blood flow through an influence of the latter on the transcapillary osmolar extraction. An attempt to approach these problems was made by investigating the relationships between the fluid flux and the veno-arterial osmolar difference and blood flow respectively and by a theoretical analysis of the result given in the Comments section. The observations were made in early phases of exercise to minimize interference with the fluid movement from a later gradual tissue pressure increase.

Fig. 22 is a representative original record from a series of experiments (n = 4) indicating that the net transcapillary fluid movement during exercise is directly related to the transcapillary osmotic gradient. In these experiments, performed under constant flow perfusion, vascular tone was completely abolished by a continuous i.a. infusion of a supramaximal dose of papaverine. Thus superimposed exercise could not elicit any further changes of vascular tone and, hence, no further increase of hydrostatic capillary pressure. Net fluid *filtration* induced by exercise *per se* was thus prevented. Since blood flow and CFC were constant throughout the experiment (Fig. 22), hemodynamic variables of possible importance for osmotic fluid exchange, such as blood flow capillary flow distribution, capillary flow velocity capillary surface area and capillary permeability could be considered constant (see Comments) Under these experimental conditions, the degree of extravascular hyperosmolality was varied by performing

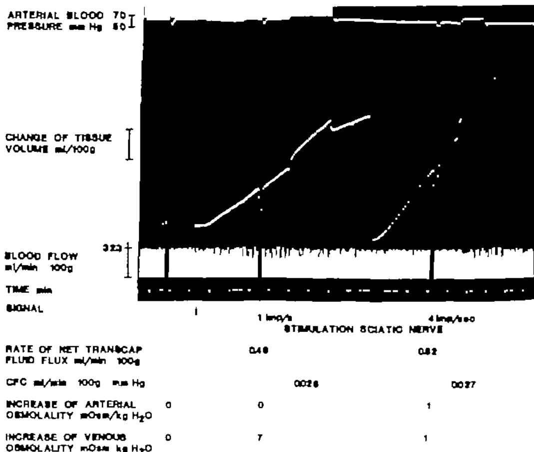


Fig 22 Experiment indicating that osmotic fluid flux to exercising muscle is proportional to th induced veno-arterial osmolar d fference which was varied by performing work at two different intensities (somato-motor fibre stimulation 1 and 4 imp/sec). Vascular tone during the experiment abolished by continuous a. infusion of papaverine so that exercise *per se* could not elicit any change of capillary hydrostatic pressure, capillary surface area (CFC) or fluid filtration

work at two different intensities (somato-motor fibre stimulation at 1 and 4 imp/sec) The veno-arterial osmolar difference induced by work was determined at the two levels of exercise. Under the prevailing experimental conditions, this difference may be directly related to the evoked transcapillary osmolar gradient (see Comments) It can be seen from the figure that stimulation at 1 imp/sec caused an osmotic transcapillary fluid flux which, after about 1 minute, occurred at an approximately steady rate of 0 46 ml/min x 100 g tissue. The induced veno-arterial osmolar difference, determined after about 3 minutes, was 7 mOsm. When the stimulation rate was increased to 4 imp/sec, the maximal fluid flux occurred at a rate of 0 82 ml/min x 100 g and concomitantly the induced veno-arterial osmolar difference was increased to 13 mOsm. The rate of net transcapillary fluid movement was about 1 8 times greater at the higher work intensity

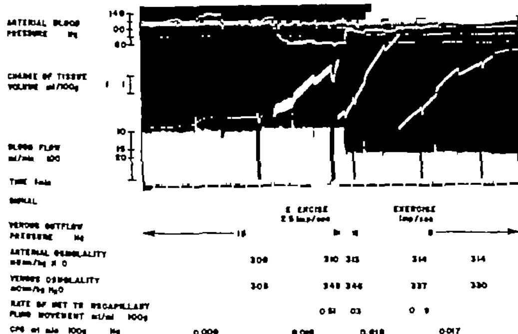


Fig 23 Experiment indicating that the net transcapillary osmotic fluid flux to exercising muscle is proportional to blood flow under conditions of constant veno-arterial osmolar difference. For explanation see text.

as was the induced veno-arterial osmolar difference The fluid flux was thus directly proportional to the osmolar difference and therefore probably also to the transcapillary osmotic gradient (see above) The other experiments in this series supported this conclusion These findings might further suggest that the substances responsible for tissue hyperosmolality at the different work intensities had roughly similar transcapillary reflection coefficients (see Comments)

Fig. 23 shows original tracings from a series of model experiments (n = 4) suggesting that the magnitude of the net transcapillary osmotic fluid flux to exercising muscle is directly related to the volume flow of blood under conditions of a constant veno-arterial osmolar difference. The osmotic fluid flux (tissue volume increase) was thus studied at two levels of blood flow in this case 9.3 and 18 ml/min x 100 g tissue, flow being controlled by a perfusion pump To be able to maintain an approximately constant veno- arterial osmolar difference at both these flow levels, the somato-motor fibre stimulation frequency had to be raised somewhat (from 2 5 to 4 imp/sec) during the larger flow It is most likely that this had to be done to compensate for an increased "osmolar washout" from the tissue (decreased interstitial osmolality) and for a somewhat decreased transcapillary osmolar extraction. CFC during exercise was of similar magnitude at the two flow levels (Fig 23) indicating that functional

capillary surface area and capillary flow distribution were kept approximately constant. Finally significant variation of capillary hydrostatic pressure and thus of net fluid filtration at the two different blood flows was avoided by appropriate adjustments of venous outflow pressure. Thus, at the lower blood flow venous outflow pressure was set at 15 mm Hg (in control state as well as during exercise at 2.5 imp/sec) and was lowered by 10 mm Hg when flow was increased in order to compensate for the concomitant rise of arterial inflow pressure of about 35 mm Hg Assuming a ratio of pre- to post capillary resistance of 3 1 mean hydrostatic capillary pressure can be calculated to be about 26 mm Hg at both flow rates. It can be seen that at the lower blood flow exercise led to a net transcapillary fluid flux which after the CFC determination reached a maximal rate of 0.51 ml/min x 100 g tissue at which time the veno-arterial osmolar difference was 35 mOsm. When flow was raised (approximately by 100 %) there was an abrupt increase of the rate of net transcapillary fluid movement to 1.03 ml/min x 100 g which was then maintained constant for some minutes. The veno-arterial osmolar difference was almost the same as before (33 mOsm) The fact that a twofold increase of blood flow was associated with a twofold increase of the osmotic fluid flux suggests that the latter is directly related to volume flow of blood under conditions of constant veno-arterial osmolar difference. The other experiments in this series supported this conclusion (Note in this connection that the results above indicated that the substances responsible for tissue hyperosmolality had similar transcapillary reflection coefficients at different stimulation rates)

In later phases of the work (Fig 23) it can be seen that the net transcapillary fluid movement gradually declined with time. This can mainly be ascribed to a decreasing transcapillary osmolar gradient reflected in the lower veno-arterial osmolar difference (see below) Further a gradual increase of tissue pressure could have contributed to this effect.

The experiments depicted in Figs. 22 and 23 thus indicate that the osmotic net trancapillary fluid movement during exercise may be directly related both to the veno-arterial osmolar difference and to blood flow Therefore it would appear that in the individual exercise experiment the osmotic fluid flux is directly related to the product of veno-arterial osmolar difference and blood flow This was analysed by plotting osmotic flux versus the above-mentioned product (Fig 24) for all exercise experiments (n = 60) performed both when flow was free to change (average data previously discussed, Fig. 21) and when it was controlled with a perfusion pump (experiments examplified in Figs. 22 and 23) The data were obtained after about 2—4 min of work of different intensities when the rate of transcapillary fluid movement was maximal. For the free flow experiments, osmotic fluid flux was calculated by subtracting net fluid filtration (capillary hydrostatic pressure increase x CFC) from the observed total fluid flux. It was assumed, as suggested in Chapter III that capillary hydrostatic pressure was about unchanged during light exercise, and rose by 4 and 8 mm Hg

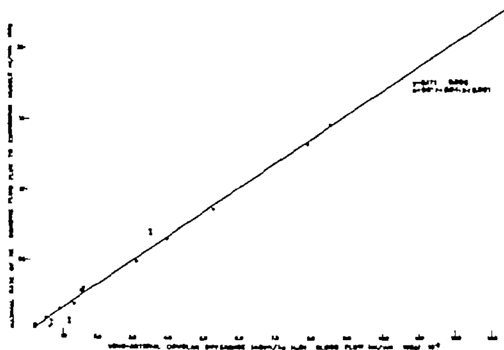


Fig 24 Diagram of the maximal rate of net transcapillary osmotic fluid transfer into exercising muscle observed t diff rent work intensities (sciatic nerv stimulation at 0.25—4 imp/sec) against the product of induced veno-arterial osmolar difference × blood flow × 10^{-3} Data indicate a direct relationship between the plotted parameters.

during moderate and heavy exercise respectively (The other experiments were designed so that the osmotic fluid flux was obtained directly see above) It can be seen from the diagram that the osmotic fluid flux was related to the calculated product and linear regression analysis indicated a direct relation ($r = 0.94$ $p < 0.001$) The regression line intersects the vertical axis at a point near the origin. Spread of data in the diagram might be entirely due to difficulties in determining the measured variables exactly A non linearity however should be present if solutes with different transcapillary reflection coefficients were responsible for the interstitial hyperosmolality at different work intensities. This possibility seemed less likely however since the osmotic fluid flux per unit veno-arterial difference x flow showed no statistically significant correlation to the rate of somato-motor fibre stimulation, an observation which supported the earlier conclusion reached from the type of experiment described in Fig 22. That indeed a linear relation between the variables in Fig 24 should be present is supported by the analysis given in Comments section The regression equation found in Fig. 24 will be used in an attempt to distinguish the hydrostatic and osmotic forces involved in the fluid accumulation in muscle during prolonged work and in the disappearance of accumulated fluid in the postexercise period These problems will be considered in the following sections.

During prolonged" exercise periods (15 min), the hyperemia (vascular conductance) and the CFC usually remained approximately constant of the levels observed in the early phase (<4 min) Sometimes, however CFC determinations performed in late periods of prolonged heavy work showed somewhat lower values than in early periods, but this may merely reflect interference on the determination from a gradual increase of tissue pressure (*cf* Jacobsson and Kjellmer 1964) The rate of net transcapillary fluid movement was relatively constant throughout light exercise of 15 min duration but it declined gradually with time during heavy exercise. These findings may mainly be explained by the fact that the transcapillary osmotic gradient, as reflected by the veno-arterial osmolar difference, remained approximately constant during light exercise, but showed a gradual decrease during heavy work (*cf* Fig 23) Thus, the arterial osmolality as well as the venous hyperosmolality remained roughly constant throughout light exercise whereas, during heavy exercise, arterial osmolality tended to increase and venous hyperosmolality tended to decrease with time (*cf* Chapter II Results section A 1)

Fig 25 shows collected mean data ± SEM (n=9) on fluid accumulation (left ordinate) in muscle during prolonged heavy exercise and on the evoked changes of the veno-arterial osmolar difference (right ordinate) the latter determined 3 7 and 15 min after the commencement of work (correction was here made for the initial capacitance response which averaged 0.58 ml/100 g tissue). It can be seen that the rate of transcapillary fluid movement was maximal in the time period some 1 to 4 min after beginning of work and that it then decreased gradually After 15 min of work, the total amount of accumulated fluid averaged 14 8 ml/ 100 g tissue. The induced veno-arterial osmolar difference averaged 17 0 mOsm after 3 min 9 4 mOsm after 7 min, and 3.3 mOsm after 15 min of work. These data strongly suggest that the gradual decline of the rate of fluid accumulation in the exercising muscles to a great extent can be ascribed to the successive decrease of the veno-arterial osmolar difference (reflecting the trancapillary osmolar gradient)

It is conceivable, however that the pronounced increase of extravascular fluid volume could lead to some increase of tissue pressure which also would tend to counteract the transcapillary fluid flux. The following deductions may permit a rough estimation of the extent to which tissue pressure changed on the average, in the experiments depicted in Fig 25 CFC averaged 0 037 ml/min x 100 g x mm Hg in early phases of work. Assuming, as discussed above, that mean capillary hydrostatic pressure during heavy exercise rises by 8 mm Hg, the resulting net fluid filtration at 3 min of exercise would amount to 0 30 ml/min x 100 g, if no significant tissue pressure changes were present at this time. The average rate of total fluid flux at 3 min was 1 92 ml/min x 100 g (calculated as the mean of the fluid flux during the 3rd and 4th min) and the osmotic fluid flux therefore was 1 62 ml/min x 100 g The latter figure agrees well with the osmotic flux deduced from the regression line of Fig 24 which showed the

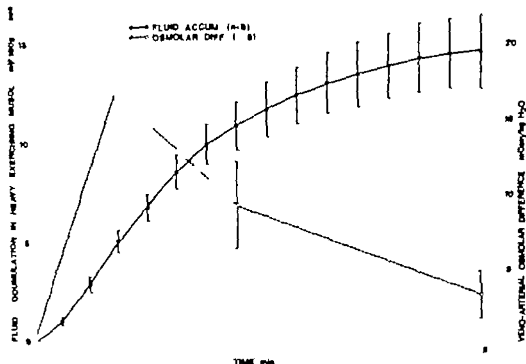


Fig 23 Diagram showing mean data ± SEM (9 exp.) on the fluid accumulation in skeletal muscle (left ordinate) during 15 min of heavy exercise (somato-motor fibre stimulation 4 imp/sec) and on the concomitant work induced veno-arterial osmolar difference (right ordinate), the latter determined 3 7 and 15 min after commencement of work.

relation between the osmotic fluid flux and the product of veno-arterial osmolar difference times blood flow for short term exercise. Thus this product was 8 98 x 10³ at 3 min (blood flow averaged 52.8 ml/min x 100 g) giving a value for osmotic fluid flux of 1 53 ml/min x 100 g If a linear relationship between *the osmotic fluid flux and this product (P) were present throughout* prolonged work the osmotic fluid flux at 7 min (blood flow averaged 43 7 ml) can be calculated as (P *7 min/P 3 min)* x osmotic fluid flux *3 min* This gives a value of 0.74 ml/min x 100 g ((4 11 x 10³/8 98 x 10³) x 1 62) A similar deduction for osmotic fluid flux at 15 min (blood flow averaged 38 8) *gives a value of 0.23 ml/min x 100 g ((1.28 x 10³/8 98 x 10³) x 1 62) (it may* be mentioned that the somewhat reduced hyperemia with time during the exercise largely could be attributed to a decrease of the perfusion pressure) The capillary hydrostatic pressure rise and CFC should be roughly similar throughout the prolonged exercise and the rate of net transcapillary fluid filtration, in turn should therefore be about constant, or 0.30 ml/min x 100 g, *if no change of tissue* pressure occurred. Theoretically therefore, the rate of total fluid flux at 7 and 15

min of work should be 1 04 (0 74+0.30) and 0 53 (0.23+0.30) ml/min x 100 g, respectively The observed rates of total fluid flux in these periods, however averaged 0 84 (mean of fluid flux during the 7th and 8th min) and 0 13 ml/min x 100 g (fluid flux during the 15th min) i.e 0.20 and 0 40 ml/min x 100 g less than that theoretically expected if there were no change of tissue pressure. A value for the increase of tissue pressure may be obtained by dividing these latter values by CFC (0.20/0 037 0 40/0 037) thus indicating that tissue pressure had increased by 5 mm Hg at 7 min and by 11 mm Hg at 15 min of exercise Admittedly these calculations are crude and indirect but nevertheless the figures for the tissue pressure change, after correction for the effects of some increase of plasma colloid osmotic pressure, are in close agreement with reported findings obtained with an independent method (see Comments)

After cessation of exercise, tissue volume slowly and gradually decreased towards the resting control level prevailing before work The time required for this return of tissue volume was related to the amount of accumulated fluid at the end of the exercise period. After prolonged (15 min) heavy exercise, when the fluid accumulation could exceed 20 ml/100 g tissue, tissue volume was sometimes not restored until 4 hours had elapsed The rate of tissue volume decline was most rapid in the initial period after the cessation of exercise and decreased with time.

In an attempt to analyse the mechanisms responsible for the fluid absorption after exercise, tissue volume, blood flow CFC, and arterial and venous osmolality were followed after prolonged (10—15 min) heavy work (6 animals) The mean results ± SEM are shown in Fig. 26 In the diagram, the disappearance of the fluid accumulated in the muscles during exercise (left ordinate) and the arterio-venous osmolar difference (right ordinate) in the post-exercise period are plotted against time. Other calculated, variables are shown below the diagram. It can be seen that the accumulated fluid, which averaged 14 8 ml/100 g at the end of the exercise period disappeared at gradually declining rates over the time period of 3 hr at the end of which there was still some of the accumulated fluid present (3 ml/100 g tissue)

A constant observation was that the veno-arterial osmolar difference present during work (see Fig 25) was reversed in the post-exercise period implying that after exercise arterial osmolality in fact exceeded venous osmolality (i.e there was a transcapillary osmolar gradient from blood to tissue) As can be seen from Fig 26 the average negative arterio-venous osmolar difference of 7 mOsm at the end of work was reversed already 5 min after the cessation of exercise to a positive value of some 2 mOsm The arterio-venous osmolar difference gradually rose to a plateau value of about 4 mOsm in a time of between 20 — 80 min after which it again declined. The reversal of the arterio-venous difference after work was due to declining venous osmolality whereas the arterial osmolality which was raised during the prolonged work remained elevated for a considerable length of time after exercise. It is conceivable that the higher blood

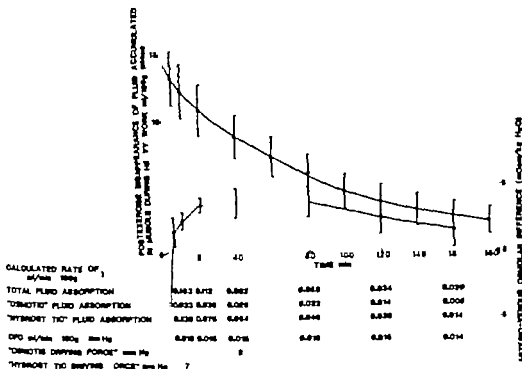


Fig 26. Diagram showing mean data ± SEM (6 exp.) on post-exercise disappearance of fluid accumulated in muscle during work (left ordinate) and of the concomitantly observed arterio-venous osmolar difference (right ordinate). Derived variables shown t bottom of figure.

than tissue osmolality in the post-exercise period could cause an osmotic fluid absorption and partly explain the fluid disappearance· (Note that in these ex periments lymph drainage was prevented implying that fluid disappearance solely occurred as a transcapillary process) It seems that the importance of an osmotic fluid flux, caused by solutes other than plasma proteins, might be revealed from values for blood flow and arterio-venous osmolar difference if a linear relationship between the product of these two factors and osmotic fluid flux (like in Fig 24) could be established also with regard to transcapillary fluid absorption. That this is so is strongly suggested by the data given in Fig. 27 which is based on results obtained during intra-arterial hypertonic glucose infusion to the resting skeletal muscle. The data refer to constant pressure per fusion experiments and the hypertonic infusions were adjusted so as to elicit varying rates of fluid absorption and hyperemia responses. (A possible decrease of tissue pressure was considered roughly balanced by the concomitant rise of capillary hydrostatic pressure during hypertonic infusion, *cf* Chapter III) It can be seen that the equations for the regression lines in this figure and in Fig. 24 are quite similar Calculation of osmotic fluid absorption from either equa tion would thus give similar results. That of Fig. 24 is used below because its regression line, as should be expected, passes through the origin (or nearly so)

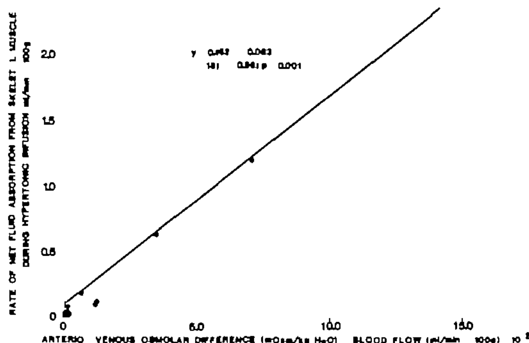


Fig 27 Rate of net transcapillary fluid absorption in skeletal muscle caused by i. a. infusion of hypertonic glucose solution plotted against the product of the simultaneously determined arterio-venous osmolar difference × blood flow × 10^{-2} A direct relation seems present.

and because it represents osmotic flux caused by solutes present in the exercise and probably also in the post-exercise period (see Comments)

The data presented below the diagram in Fig. 26 shows for the post-exercise period the following deduced variables The rates of total fluid absorption *(obtained from tangents drawn to the smooth absorption curve) of osmotic* fluid absorption (obtained from the depicted average values for arterio-venous osmolar difference and from average blood flow values using the regression equation in Fig 24) and of fluid absorption caused by transcapillary hydrostatic (including colloid osmotic) forces (difference between total and osmotic fluid absorption) further observed mean CFC values and calculated transcapillary "driving forces" are shown Data on fluid absorption rates are given at intervals from the 10th min after cessation of work At this time the post-exercise hyperemia and therefore the co-ordinated regional blood volume change, had disappeared which implied that the tissue volume decline after this period could be entirely ascribed to net transcapillary fluid movement The calculated data in Fig 26 suggest that the fluid absorption was mainly due to transcapillary hydrostatic and colloid osmotic forces osmotic absorption [illegible] by smaller

molecules might also be quite significant, being responsible for 20 to 40 per cent of total fluid absorption. By dividing the calculated rates of transcapillary fluid absorption by the CFC, the depicted values for effective transcapillary "hydrostatic and osmotic" driving forces were obtained. Note that the early value for hydrostatic force agrees quite well with the values for "tissue pressure" increase during exercise arrived at in Fig. 25.

COMMENTS

The aim of this study was to analyse quantitatively the net transcapillary fluid movement in skeletal muscle during and after exercise and to elucidate the mechanisms responsible for this transport. To be able to study transcapillary events only, possible lymph drainage had to be excluded. This was done by ligation and cauterization of the regional lymph vessels. The significance of such interference with lymph drainage for the fluid accumulation and disappearance during and after work will be considered later on.

As early as 1915 Barcroft and Kato postulated from indirect measurements that the transcapillary fluid flux into exercising muscle must be ascribed not only to increased capillary hydrostatic pressure but also to osmosis due to metabolically increased tissue osmolality. This opinion received further support from later measurements of regional changes of vapor pressure during exercise (for details see review by Landis 1934). On the other hand, in a recent study (Kjellmer 1964 *a*), in which transcapillary fluid movement into exercising muscle was measured more directly, it was concluded that the fluid transfer was caused solely by filtration due to an increased capillary hydrostatic pressure. This conclusion was mainly based on the finding that mechanical reduction of arterial inflow pressure, and hence of capillary pressure, could lead to abolition of the transcapillary fluid flux in exercising muscle. Since this procedure, in addition, caused pronounced reduction of regional blood flow, and since the present results indicate that osmotic fluid flux in exercise is flow dependent (see further below), the osmotic mechanism for fluid transfer might easily have been overlooked in that type of experiment.

In the present study, as in that of Kjellmer, net transcapillary fluid movement into muscle was followed by continuous volumetric recording of changes in tissue volume. With this technique it is possible to distinguish between alterations of intra- and extravascular fluid volumes (see Methods). From the present investigation of concomitantly occurring fluid transfer, changes of capillary hydrostatic pressure, transcapillary hydrodynamic conductivity (CFC) and regional osmolar events it seems clear that the fluid accumulation in exercising muscle is mainly due to transcapillary fluid osmosis resulting from regional tissue hyperosmolality. Thus, at all work intensities, more than 75 per cent of the transcapillary fluid flux could be ascribed to fluid osmosis, the remaining part being due to fluid filtration caused by increased capillary hydrostatic pressure (*e.g.* Fig. 21). This estimation was derived from analyses performed in the phase of maximal

fluid transfer rate occurring early in exercise but appears representative also for later stages of work (as can be deduced from the data in Fig 25) To exemplify that the fluid flux can by no means be explained by filtration alone, it may be mentioned that the fluid transfer sometimes was so rapid as to require a rise of mean capillary hydrostatic pressure of nearly 100 mm Hg (*e g* panel A, Fig. 20). This value far exceeds the capillary pressure rise that can occur in exercising muscle at heart level. As indicated by the present results, the capillary pressure increase at most can amount to 10 to 15 mm Hg The only reasonable conclusion is that the pronounced tissue hyperosmolality is responsible for the major part of the fluid transfer

During prolonged work (15 min duration) the net transcapillary fluid movement tended to decline gradually with time, a feature most prominent during heavy work (Fig 25). This decline could be ascribed mainly to a successive decrease of the transcapillary osmolar gradient, due partly to some increase of arterial osmolality and partly to a decrease of the interstitial (venous) hyperosmolality To some extent the declining rate of fluid flux seemed also related to a gradual increase of tissue pressure The increase of arterial osmolality can be explained, at least partly by a "delivery of osmols" from the exercising muscles to the general circulation and the decline of interstitial hyperosmolality partly by a possible decrease of the "osmolar production" in the active muscles and partly by a regional osmotic dilution" as tissue fluid volume increases.

The magnitude of the tissue pressure changes caused by the fluid accumulation in exercising cat calf muscles has previously been evaluated by Kjellmer (1964 *b*) Since tissue pressure seemed to vary between different portions of the muscles his data were given in terms of a minimal and a maximal pressure value. An increase of extravascular tissue volume by 10 to 15 per cent caused tissue pressure to rise by some 3 to 6 mm Hg (deduced from Fig 7 in Kjellmer's paper) Rough deductions from the present data (Fig 25) suggested that "tissue pressure" rose by some 5 to 11 mm Hg at a comparable increase of tissue volume (10 to 15 per cent) but this estimation also included the effect of a possibly altered transcapillary colloid osmotic pressure gradient. The effect of changes of the interstitial protein concentration during exercise would appear almost negligible since the resting protein concentration is low and since a significant part of the fluid transfered into the working muscles apparently accumulates inside the striated muscle cells (see below) The effect of altered plasma protein concentration, on the other hand may be significant due to the relatively high concentration at rest and to the disproportionate increase of plasma colloid osmotic pressure as a function of plasma fluid loss (see Landis and Pappenheimer 1963) From the observed plasma fluid loss it can thus be calculated that plasma colloid osmotic pressure on an average might have risen by about 2 mm Hg towards the end of the exercise experiments depicted in Fig 25 The present data may therefore be taken to indicate a tissue pressure increase during prolonged exercise of some 3 to 9 mm Hg which is relatively close to Kjellmer's

findings (1964 *b*). Mention should finally be made that when resting skeletal muscle volume was increased by 15 per cent, Guyton (1965) found tissue pressure to rise by about 6 mm Hg from a negative value in the control period

The total amount of fluid that accumulated in the cat lower leg muscles during 15 min of heavy work (4 imp/sec) averaged as mentioned above, no less than about 15 ml/100 g tissue (Fig. 25) which is in agreement with the highest values reported by Jacobsson and Kjellmer (1964) More than 60 per cent of this "oedema" was formed within the first 5 min of work. Under normal *in vivo* conditions, the fluid accumulation would be less pronounced due to fluid drainage *via* the intact lymph system. Jacobsson and Kjellmer (1964) who studied the cumulative outflow of lymph from a catheterized popliteal main lymph vessel in the cat during a 20 min period of work of the calf muscles found, however that it was quite small and no more than a total of 0 1 ml. Since other lymph vessels in the knee region were ligated in that study total lymph drainage may be somewhat larger in the intact organism Nevertheless, these data suggest that lymph drainage from working muscle is quite small and, hence, that the fluid accumulation in the active muscle may not be much less under normal circumstances than found in the present study Perhaps the surprisingly small lymph flow is explained by the fact that a considerable part of the fluid transfered from the blood stream must accumulate in the intracellular space of muscle (see below).

It should be stressed that the present data on fluid accumulation in muscle refer to work performed with a relatively small part of the total muscle mass of the animal. In situations where the major part of the muscle mass in the body is active, the fluid accumulation per unit tissue must be far less since it can be calculated that, otherwise, virtually all plasma fluid would be lost within some 5 min of heavy work. Under these circumstances, mechanisms which limit the plasma fluid loss must therefore be put into play This problem which was approached in a recent study (Lundvall *et al* 1970, 1972) will be discussed below (General discussion)

The tissue hyperosmolality in exercising muscle must primarily be created inside the contracting striated muscle cells as a result of the increased tissue metabolism. A significant, perhaps major part of the osmotically transfered fluid from the blood stream can therefore, as already mentioned, be expected to accumulate in the intracellular space of striated muscle. This opinion is supported by a recent study by Bergström *et al* (1971) who estimated changes of extra and intracellular water content in muscle evoked by prolonged (20 min) moderate to heavy work in man. On the average, total muscle water calculated in ml/100 g fat-free solids, increased by 21 per cent and the increased water content was estimated to be roughly equally distributed between the intracellular and interstitial spaces.

The fluid volume that accumulates in the skeletal muscle during exercise, regardless of its final distribution into the different tissue compartments, is of

course derived from the blood stream and therefore this entire fluid volume must traverse the capillary membranes Some characteristics of this capillary exchange process will be considered below

It is generally believed that plasma fluid passes the capillary barrier *via* gaps (about 40 Å pores") at the intercellular (endothelial) junctions but, as suggested by recent findings in heart muscle, water may also to some extent traverse the capillary endothelial cells themselves (Yudilevich and Alvarez 1967). However the quantitative relation between fluid movements *via* these routes is so far unknown.

Evidence was presented above that the fluid flux to the extravascular space of exercising muscle was partly due to a filtration process and partly to an osmotic process. Net fluid filtration into the tissue occurs at a rate determined by the product of the increase of the transcapillary hydrostatic pressure gradient and the prevailing transcapillary hydrodynamic conductivity the latter being measured for the whole region in terms of CFC in the present study Data on these quantities were presented above and in addition, a rough evaluation of secondary changes of the transcapillary colloid osmotic pressure gradient was made. The osmotic transcapillary fluid flux into the exercising muscle (primarily into its interstitium) is a more complex process influenced by a multitude of factors. The results presented in Chapter II indicated that the interstitial hyperosmolality in exercise can be ascribed to increased concentrations of several substances, in early phases especially of sodium and lactate, but also of others such as potassium etc. The increased concentrations of most of these agents in the interstitium would seem mainly to be caused by release of osmotically active products from the contracting muscle fibres the osmotic transfer of water into the intracellular (hypertonic) space of muscle must also contribute to raised interstitial solute concentrations (*e g* with regard to sodium) The evoked osmotic transcapillary fluid flux should be governed among other things, by the transcapillary concentration (osmolar) gradients for these different substances, by their transcapillary osmotic reflection coefficients, and by the capillary hydrodynamic conductivity The present study does not, of course, permit an assessment of the complex interplay between all individual factors determining the osmotic fluid flux during exercise but nevertheless, the present analysis (see Figs. 22—24) may lead to some conclusions about certain basic characteristics of this process.

Before discussing these results, some factors of importance for the capillary exchange will be considered CFC is a measure of the total transcapillary hydrodynamic conductivity in the region and is, in turn a function of the specific capillary permeability (per unit area) and the prevailing capillary surface area available for fluid exchange (*e g* Folkow and Mellander 1970) Since there is no indication that capillary permeability in muscle is altered by exercise (Arturson and Kjellmer 1964) changes of CFC would reflect alterations of the functional capillary surface area, as caused by changes of the number of patent

(perfused) capillaries and controlled by the activity of the precapillary "sphinc ters" (cf Mellander and Johansson 1968). Although the bore and length and thus the surface area of individual capillaries vary considerably differences in CFC will provide information about changes in the number of patent, average" ca pillaries because of the inclusion of a vast number of capillaries on a random basis in every measurement. It follows that relative changes of capillary flow velocity will be reflected by changes in the ratio of blood flow/CFC.

Experimental evidence (Fig. 22) indicated that the osmotic fluid flux was di rectly proportional to the veno-arterial osmolar difference (varied by perfor ming work at different intensities) under conditions when blood flow and CFC were kept constant and therefore, capillary surface area, capillary flow distribution, capillary flow velocity and capillary permeability could be considered constant (cf also Lundgren and Mellander 1967) Further under conditions of approximately constant veno-arterial osmolar difference and CFC, the osmotic fluid flux was directly proportional to the volume flow of blood (Fig. 23) and, hence, to capillary flow velocity The collected data from exercise experiments of different intensities (light to heavy work) in which blood flow CFC and veno-arterial osmolar difference varied within wide ranges, indicated that the osmotic fluid flux was directly proportional to the product of the veno-arterial osmolar difference and volume flow of blood (Fig. 24)

The interpretation of the present results may be aided by the theoretical ana lysis below although this analysis certainly implies that the osmotic fluid ex change process in viewed in a simplified way For this analysis the following assumptions were made· 1) The hyperosmolality in the interstitium of the exer cising muscle is considered uniform (well mixed tissue compartment) without any significant osmolar gradient along the external side of the capillary This assumption may be justified for several reasons. All muscle fibres were contracting (supramaximal somato-motor fibre stimulation) thus "delivering osmols" throughout the interstitium. The extensive anastomosing in the capillary net work (e g Wiedeman 1963) tends to minimize interstitial solute concentration gradients. Fur ther the fluid streaming into the interstitial space during work might have a "stirring" effect (cf Lundgren and Mellander 1967) 2) Solute and fluid permea bility is considered roughly constant along the capillary The longitudinal gra dient of capillary permeability observed in some tissues (e g Zweifach and Intag lietta 1968) has so far not been clearly demonstrated in skeletal muscle (Smaje *et al.* 1970).

Fig. 28 depicts the osmolality increase in blood passing a single idealized unit" capillary in exercising muscle. L denotes the total length of the capillary and x distance from its orifice on the arterial side· ΔOsm (0) denotes the osmololity difference between the interstitial space and the blood at the arterial orifice, and ΔOsm (x) the osmolar difference at distance x from the orifice. ΔOsm (V A) denotes the measured veno-arterial osmolar difference. For a stagnant column of capillary blood, the decrease of the transcapillary osmolar difference, caused

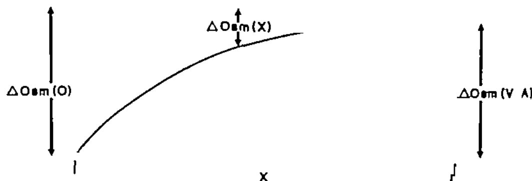


Fig. 28 Schematic illustration of osmolality increase in blood passing a single idealized unit capillary in exercising muscle. L denotes the total length of the capillary and x distance from the orifice on the arterial side. Δ Osm (o) denotes the osmolality difference between the interstitial space and blood at the arterial orifice and Δ Osm (x) the osmolality difference at distance x. Δ Osm (V—A) denotes determined veno-arterial osmolar difference.

by solute diffusion from the interstitial space and fluid transfer in the opposite direction, may be described by the differential equation

$$\frac{d(\Delta Osm\ (t))}{dt} = - \frac{\Delta Osm\ (t)}{\tau} \quad (1)$$

where τ is a time constant determined by the characteristics of the substances involved in the exchange process, capillary pore size, temperature etc. The solution to equation (1) is:

$$\Delta Osm\ (t) = \Delta Osm\ (0)\ e^{-\frac{t}{\tau}} \quad (2)$$

If blood moves through the capillary with a constant velocity v equation (2) yields

$$\Delta Osm\ (x) = \Delta Osm\ (0)\ e^{-\frac{x}{v\ \tau}} \quad (3)$$

where $t = \frac{x}{v}$ *i.e.* distance/(distance/time)

By integration over the entire length of the capillary the mean transcapillary osmolar difference is obtained.

$$<\Delta Osm\ (x)> = \frac{1}{L}\int_0^L \Delta Osm\ (x)\ dx = \Delta Osm\ (0)\ \frac{v}{L}\ (1-e^{-\frac{L}{v\ \tau}}) \quad (4)$$

Considering the muscle capillary bed as consisting of a number of "unit" capillaries with an overall transcapillary hydrodynamic conductivity expressed by CFC, Van t Hoff's law modified with regard to the osmotic reflection coefficient for the case of leaky capillary membranes (*cf* Landis and Pappenheimer

1963), gives the following expression of the osmotic transcapillary fluid flux J into exercising muscle in a given situation:

$$J = R\ T\ \Delta Osm(0)\ \frac{v\ \tau}{L}\ \left(1 - e^{-\frac{L}{v\ \tau}}\right)\ \sigma\ \ CFC \qquad (5)$$

In this formula J and CFC are expressed per unit muscle weight, and σ represents a "weighed mean" of the transcapillary osmotic reflection coefficients for the substances responsible for the interstitial hyperosmolality of exercise (R but also T and L are constants)

In the present experiments, ΔOsm (0) could not be measured and the osmotic fluid flux was studied merely in relation to the veno-arterial osmolar difference, ΔOsm (V—A) which, according to (3) can be written

$$\Delta Osm(V-A) = \Delta Osm(0) - \Delta Osm(0)\ \ e^{-\frac{L}{v}} = \Delta Osm(0)\ (1 - e^{-\frac{L}{v\ \tau}}) \qquad (6)$$

Equation (5) thus be transformed

$$J = R\ \ T\ \ \Delta Osm(V-A)\ \ \frac{v\ \tau}{L} \cdot \sigma\ \ \ CFC \qquad (7)$$

The results presented in Figs. 22 to 24 may now be discussed in view of this latter expression. In the experiments illustrated in Fig. 22 blood flow and CFC and therefore also v (proportional to flow/CFC, see above) were kept constant. J was found to vary in direct relation to Δ Osm(V—A), changes of the latter being produced by varying the sciatic nerve stimulation frequency (work intensity). Since R, T and L are constant it follows that the product σ τ can be considered constant. Thus, at different work intensities, both σ and either were constant, or they changed equally much in opposite directions. If σ increased with increasing work intensity it would imply that the solutes responsible for the interstitial hyperosmolality exhibited a corresponding decrease of their transcapillary diffusibility According to (1) however τ must then increase concomitantly unless the fluid transfered in the osmolar exchange process, as a result of a simultaneous change of its tonicity contributed significantly more to the osmolar equilibration between tissue and blood than at low work intensities, which appears less likely The reverse would be true if σ decreased. The experimental findings, as already mentioned seemed to refute the possibility that σ and τ change in the *same* direction It may be concluded that σ as well as τ tended to be roughly constant at different work intensities. According to (6) and (4) the veno-arterial osmolar difference, in its turn, would then be proportional to the mean transcapillary osmolar difference under the conditions prevailing in the experiment of Fig. 22.

In Fig. 23 Δ Osm(V—A) and CFC were kept roughly constant while blood flow was varied. Thus v varied with blood flow and, according to (7), as did J It is clear that in this type of experiment Δ Osm(0) increased with v (see(3))

The data in Fig 24 finally indicated that J varied directly with the product blood flow times Δ Osm(V—A). This product can be written as

$\frac{flow}{CFC}$ CFC ΔOsm(V—A) or since $\frac{flow}{CFC}$ is proportional to v as a constant v CFC ΔOsm(V—A) It follows that the linear relationship indicated by the experimental data depicted in Fig 24 is in accordance with (7)

An attempt will be made below to estimate to what extent venous hyperosmolality reflects interstitial hyperosmolality induced by work, a problem of great importance for the quantitative evaluation of the role of hyperosmolality in exercise hyperemia. Another approach to the same problem was presented in Chapter II The relation between venous and interstitial hyperosmolality using the above defined terms, is given by the ratio of Δ Osm(V—A)/Δ Osm(0) which, in turn, can be expressed as Δ Osm(0) $(1-e^{-\frac{L}{V}})$/Δ Osm(0) or $(1-e^{-\frac{L}{V}})$ (see (6)) This expression can be derived from (7) by using the experimentally observed values for J CFC, and Δ Osm(V—A) and given values for σ σ as mentioned above, is here considered as the weighed mean of the individual transcapillary reflection coefficients for the solutes responsible for the work induced interstitial hyperosmolality (mainly sodium and lactate, but also others, such as potassium etc Chapter II)

Since σ could not be determined in the present experiments seemingly reasonable σ values were taken from the literature. Vargas and Johnson (1964) presented experimental evidence for heart capillaries to suggest that the reflection coefficients for small lipid insoluable molecules are far below unity for urea 0 10 and for sucrose 0 30. However these results did not receive unanimous support (e.g Pappenheimer 1970) since they seemed too low to be compatible with the concept of the "pore" dimension established by extensive studies in skeletal muscle. Yet, later theoretical calculations by Perl (1971) who took the special geometry of the capillary pores (Karnovsky 1967 1970) and the possible fluid transfer across the capillary endothelial cells (Yudilevich and Alvarez 1967) into account, gave values for σ in skeletal muscle capillaries similar to those found by Vargas and Johnson. Perl thus arrived at a value for σ for NaCl of 0 16—0 18 for urea of 0 21—0 23 and for glucose of 0.22—0.27 Interestingly this analysis led to the view that such low reflection coefficient values still were in consonance with the classical estimates of capillary "pore" size (see Landis and Pappenheimer 1963)

For the present analysis, dealing with exercise hyperosmolality created by small molecules, the weighed mean of the transcapillary osmotic reflection coefficients, in view of the discussed data, was considered to be in the range of 0.10 to 0.30 In the diagram of Fig 29 the ratio of ΔOsm (V—A)/ΔiOsm (0) is plotted against the ratio of blood flow/CFC. From what has been said the dia

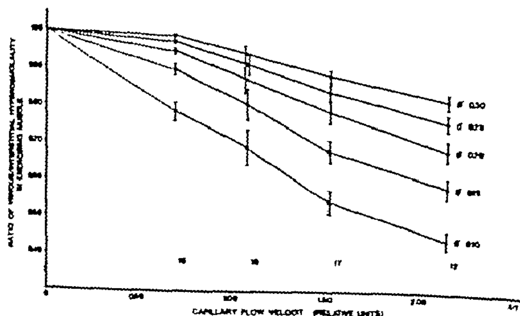


Fig. 29 Ratio of venous/interstitial hyperosmolality in exercising muscle against (blood flow/CFC) × 10⁻² The latter expression used as a relative measure of capillary flow velocity The hyperosmolality ratio is given for various values for transcapillary osmotic reflection coefficient, σ ("weighed mean" for the substances responsible for the interstitial hyperosmolality of exercise).

gram thus shows the extent to which work induced interstitial hyperosmolality is reflected in the venous effluent ("hyperosmolar extraction") at different capillary flow velocities expressed in relative units. The diagram shows calculated mean values ± SEM for 4 ranges of flow/CFC (<0.90, mean 0.71 ± 0.04 (SEM), 0.90—1.30 mean 1.09 ± 0.03, 1.30—2.00 mean 1.52 ± 0.05 and >2.00, mean 2.14 ± 0.18. All flow/CFC values have been multiplied by 10⁻²). The data indicate that for any σ value the hyperosmolar extraction is clearly below unity at the higher capillary flow rates. Note that "extraction" varies considerably with σ which for the present purpose implies that σ has to be more closely defined. An idea of the magnitude of σ can be obtained by comparing the regression lines in Fig. 24 (exercise experiments) and Fig. 27 (hypertonic glucose infusion). The fact that the slope of these two regression lines were similar may reflect that the σ value in the exercise experiments (i.e. in this case a weighed mean of several reflection coefficients) and in the infusion experiments was roughly the same. Yudilevich (1971) concluded that the σ value for glucose in skeletal muscle was about 0.25 and this value might thus, from what has been said, be valid also for the exercise situation. Fig. 29 may therefore be taken to suggest that in the present exercise experiments the "hyperosmolar extraction" varied from about 97 per cent at low flow down to about 75 per cent at high capillary flow velocities, implying that the interstitial hyperosmolality during work would have exceeded the plasma

perosmolality of the venous effluent by some 3 to 30 per cent. Although capillary flow velocity varied considerably in the individual experiments, the average flow velocity was found to be comparable for light, moderate and heavy work (*cf* data for blood flow and CFC in Fig. 21) the *mean values for the ratio of* flow/ CFC ranging between 1 19 $\times 10^3$ (light work) and 1.33 $\times 10^3$ (heavy work). As can be deduced from Fig. 29 the evaluation of interstitial hyperosmolality from osmolar changes in the venous effluent may therefore have implied an underestimation by some 10—15 per cent on an average, regardless of work intensity Note that this estimate of the transcapillary "hyperosmolar extraction" during exercise is in close agreement whith the conclusion reached in Chapter II (section A1) from experiments in which venous hyperosmolality in exercising muscle was determined before and during mechanical flow reduction.

The present experiments have made it quite clear that the tissue hyperosmolality of exercise leads to pronounced osmotic fluid transfer from the blood stream to the active muscle. In this connection it may be mentioned that considerable transcapillary osmotic fluid shifts have been reported in second degree burns, caused by a secondary developed pronounced tissue hyperosmolality in skin tissue (Arturson and Mellander 1964) In a recent study by Landis and Sage (1971) osmotic fluid transfer across single frog mesenteric capillaries was analysed in response to external hyperosmolality produced by irrigation with hypertonic solutions of among other substances, NaCl, sodium lactate and urea. These authors found that the transcapillary osmotic fluid shifts were small in relation to the hypertonicity produced and suggested that the transcapillary osmotic reflection coefficients for these substances were in the range of 0 01—0 04 thus far lower than those which may be representative for mammalian skeletal muscle capillaries. This discrepancy may simply be explained by the well-known ultrastructural differences between mesenteric and muscle (as will as skin) capillaries (e g Majno 1965), the mesenteric ones apparently being much more permeable. It can be argued, however that the osmotic reflection coefficients for mesenteric capillaries might have been somewhat underestimated in the study of Landis and Sage *due to a possible underestimation of the transcapillary osmotic gradient* some solute transfer into the capillary blood at the time of osmotic flux determination thus could have occurred.

In the present experiments several hours elapsed before the fluid accumulated in the muscles during prolonged heavy work had disappeared (Fig. 26) It is conceivable, however that in the intact organism extravascular fluid drainage *via* the lymph vessels contributes to a more rapid restoration. Evidence was presented to suggest that hydrostatic, colloid osmotic as well as non-protein osmotic forces were responsible for transcapillary fluid absorption after work. The changed hydrostatic pressure gradient after prolonged work was mainly caused by raised tissue pressure but to some extent apparently also by a decrease of capillary hydrostatic pressure below the resting control level as suggested by the small vessel pressure measurements described in Chapter III. An osmotic fluid

absorption caused by substances other than proteins was indicated by the fact that in the post-exercise period arterial osmolality clearly exceeded venous osmolality indicating an "osmolar uptake" in the muscle. This process in probably related to the restoration of the energy stores and of the electrolyte balance after work and the substances taken up may partly be same ones that were lost by the muscle during exercise.

In brief summary the present study has shown that the fluid accumulation in exercising muscle is mainly due to fluid osmosis across the capillary membranes caused by work-induced tissue hyperosmolality which primarily must involve the intracellular space and secondarily affects the interstitium. Thus, at all work intensities, more than 75 per cent of the transcapillary fluid transfer when maximal, was ascribed to fluid osmosis, the remaining part being due to fluid filtration caused by increased capillary hydrostatic pressure. The experimental findings indicated that the rate of osmotic fluid movement was directly related to the product of the established veno-arterial osmolar difference and blood flow and this relationship was supported by a theoretical analysis of the data. This analysis of the osmotic process suggested that a "weighed mean" of the transcapillary osmotic reflection coefficients for the substances responsible for the interstitial hyperosmolality was roughly constant at different work intensities. Therefore, the rate of osmotic fluid flux seemed directly related to the established mean transcapillary osmolar gradient and to the transcapillary hydrodynamic conductivity in the region the latter expressed by the capillary filtration coefficient (CFC) The magnitude of the transcapillary osmolar gradient, in turn showed a relation to the capillary flow velocity (expressed in relative units by the ratio of blood flow/CFC) through an influence on the transcapillary osmolar extraction. The rate of total fluid flux was correlated to the work intensity From the relationships mentioned and from the fact that the transcapillary fluid filtration is determined by the product of the increase of capillary hydrostatic pressure and CFC, this can be explained by a correlation between work intensity and tissue hyperosmolality blood flow CFC and increase of capillary hydrostatic pressure, respectively (cf Fig. 21) The main factor limiting the fluid transfer during more prolonged work periods was a *gradual* decline of the transcapillary osmolar gradient, but additional factors also contributed, i.e increased tissue pressure and increased plasma colloid osmotic pressure. Return of fluid to the circulatory system in the post-exercise period seemed caused by hydrostatic, colloid osmotic as well as by non-protein osmotic transcapillary forces, the latter being related to an *arterial* hyperosmolality created during exercise.

METHODS

25 cats (2.5—4.7 kg) of both sexes, anaesthetized as described in Chapter II were used in this study

The investigation was performed on the isolated right lower leg muscles which in some of the experiments were enclosed in a water filled plethysmograph (38°C) to permit continuous recording of tissue volume and determination of the capillary filtration coefficient (CFC) For surgical and technical details see Chapter II Arterial inflow pressure and regional venous outflow of blood were recorded continuously Experiments were performed under a constant pressure perfusion as well as under constant flow perfusion To permit intra-arterial infusions during constant pressure perfusion the arterial inflow was diverted from the ipsilateral femoral to the popliteal artery through a shunt circuit in which a "mixing chamber" was inserted to ensure thorough mixing with blood (Chapter II) When constant flow perfusion was employed a similar shunt circuit was used and blood flow was controlled by a Harvard perfusion pump.

Experiments involving vasoconstrictor fibre activation. The ipsilateral lumbar sympathetic chain was dissected free and cut centrally A bipolar platinum electrode was placed at the level of L 4—L 5 The vasoconstrictor fibres were stimulated at 5 V 5 msec, and at frequencies of 4—16 imp/sec. Atropine (1 mg/kg b w) was given to block the sympathetic cholinergic vasodilator fibres.

Isometric muscle exercise was produced by electrical stimulation of the intact sciatic nerve (5 V 0.5 msec, 1.5—4 imp/sec) These stimuli do not excite the sympathetic fibres to the muscle vascular bed (Chapter II) In most of the exercise experiments the isometrically developed muscle tension was measured by a Grass FT 10 transducer attached to the Achilles tendon. The leg was firmly fixed by pins inserted in the femur and tibia.

Intra-arterial infusions of isotonic and hypertonic (approximately 1500 mOsm/kg H_2O) glucose solutions were made at rates between 0.2—1.0 ml/min using a constant infusion apparatus.

Blood samples (<1.5 ml) were taken for plasma osmolality determinations from a T tube in the venous outflow tubing close to the popliteal vein after discarding the "dead space" fluid volume. The osmolality was measured and the sampled blood was compensated for as described in Chapter II

By means of the blood flow and blood pressure recordings the vascular resistance function was followed The capacitance function was followed by recording the changes of regional blood volume. This was accomplished by external monitoring changes in radio-activity of the red cells labelled with ^{51}Cr and by simultaneous plethysmographic recording of tissue volume (for details see Ablad and Mellander 1963 Kjellmer 1965 *a*) The radio-activity (100—450 KeV) was recorded by an external scintillation detector (sodium iodide crystal, 1 ³/₄ inches × 2 inches) fitted in a wide angle lead collimator and registered with a spectrometer connected to a scaler and a linear rate meter (Tri-carb scintillation spectrometer model 3022, Packard) operating a Rikadenki recorder (model

B-341). The standard deviation setting on the rate meter was 2 %. The radioactivity curve was calibrated with regard to regional changes of blood volume (ml/100 g tissue) by means of concomitant plethysmografic recordings of blood volume increases caused by standardized abrupt adjustments of venous outflow pressure.

Experiments in which myogenic reactions were studied. These studies were performed on the acutely denervated vascular bed of the lower leg muscles. Hypertonic infusions and measurement of plasma osmolality were made as described above. Changes of vascular resistance and of precapillary "sphincter" tone, the latter reflected by alterations of the capillary filtration coefficient (Chapter II) were followed in response to standardized changes of vascular transmural pressure. The experiments were performed under constant pressure perfusion and CFC was determined as described in Chapter II assuming that 80 % of the applied increase of venous outflow pressure was transmitted to the capillary level. Under constant pressure perfusion the CFC determination usually leads to some blood flow reduction as a consequence of the decreased perfusion pressure. Such a blood flow reduction, although small, can induce an error in the CFC determination during hypertonic infusion, since blood flow changes alter the dilution of the infused hypertonic solution in arterial blood and, as a consequence, the magnitude of the transcapillary osmotic fluid absorption caused by the infusion (*cf.* Chapter IV). Therefore such blood flow changes during the CFC determination were prevented by appropriate adjustments of arterial inflow pressure using a screw-clamp placed around the abdominal aorta. Vascular transmural pressure was varied by 25 mm Hg by simultaneous and equal changes of arterial inflow pressure and venous outflow pressure thus keeping perfusion pressure constant (*cf.* Lundvall *et al.* 1967).

RESULTS

Hyperosmolar versus vasoconstrictor fibre influence. In a first series of experiments (7 animals), performed under constant pressure perfusion, the interaction between experimental hyperosmolality and constrictor fibre activity in the resistance as well as in the capacitance vessels was studied. The constrictor effects of sympathetic stimulation (4—8 imp/sec for 3—4 min) were compared under control conditions and during hypertonic infusion (paired observations at a given rate of stimulation). The control stimulations always caused a marked and well maintained decrease of vascular conductance which averaged 74 ± 4.7 SEM per cent of the vascular conductance prior to the stimulation. Similarly a well maintained constriction of the capacitance vessels (decrease of regional blood volume) was observed in the control period. This response was partly due to an active constriction of the capacitance vessels and partly to passive elastic recoil as a consequence of a decreased transmural pressure in these vessels resulting from the resistance vessel constriction (*cf.* Öberg 1967). The magnitude of this passive effect was determined in the following way. When vascular conductance

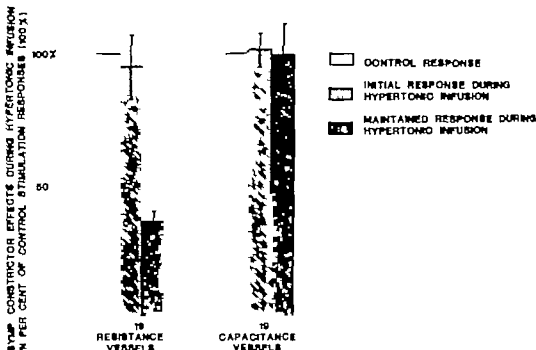


Fig 30 Diagram showing pooled data of the resistance vessel response (change of vascular conductance) and of the capacitance vessel response (change of regional blood volume) evoked by sympathetic vasoconstrictor fibre activation superimposed on i.a. hypertonic infusion (constant pressure perfusion experiments). The effects are expressed in per cent of observed control stimulation responses.

and regional blood volume had returned after the stimulation period to the control levels, blood flow was mechanically reduced to the same low level as observed during the sympathetic nerve activation The resulting passive decrease of blood volume is an approximate measure of the passive capacitance response during nerve stimulation (Mellander 1960 Öberg 1967) The blood expulsion caused by *active* constriction of the capacitance vessels could then be calculated as the total minus the passive capacitance response and it averaged 0.29 ± 0 05 SEM ml/100 g tissue, a figure in agreement with the findings of Öberg (1967)

The hypertonic infusions evoked the typical pattern of blood flow response described in Chapter II A 2, *i.e* an initial peak flow increase followed by a somewhat less pronounced steady state hyperemic phase During the steady state response to hypertonic infusion vascular conductance averaged 250 per cent of control vascular conductance and venous osmolality was raised by 25—40 mOsm/kg H_2O The sympathetic stimulation was superimposed on this steady state response to hypertonic infusion and evoked a characteristic flow response in terms of a marked initial flow decrease after which, during continued stimula tion, flow increased again to attain a steady but much less pronounced level of

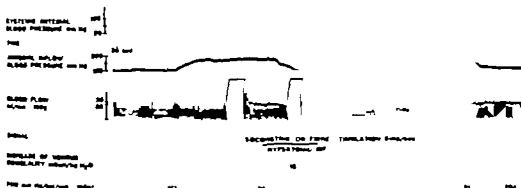


Fig. 31 Record illustrating the inhibitory effect of experimental hyperosmolality" (i.a. hypertonic glucose infusion) on sympathetic resistance and constriction during constant flow perfusion.

flow reduction. This pattern of flow response is very similar to that observed when sympathetic constrictor fibre activation is superimposed on exercise (Kjellmer 1965 *a*, *cf* also Fig. 33 below) In contrast to the transient resistance response to sympathetic stimulation the simultaneous constrictor effect in the capacitance vessels was well maintained during the period of stimulation, as shown by a sustained decrease of the regional blood volume ([51]Cr technique) After cessation of the nerve stimulation blood flow and blood volume returned to the pre-stimulatory levels. Then, during continued hypertonic infusion, blood flow was mechanically reduced to the levels observed during constrictor fibre stimulation to permit calculation of the active capacitance response to nerve stimulation (see above)

The results from the described experiments are summarized in Fig. 30 in which the sympathetic constrictor effects in the resistance vessels and the capacitance vessels (active capacitance response) during hypertonic infusion are expressed in per cent of the corresponding control stimulation effects (100 %). The diagram shows mean data ± SEM for 19 stimulation experiments. It can be seen that the initial (transient) neurogenic constrictor response of the resistance vessels during hypertonic infusion was almost as large as the control response, whereas the maintained constrictor effect averaged only 37 per cent of the control response. The active neurogenic constrictor response of the capacitance vessels, on the other hand, was not decreased during hypertonic infusion. These data thus show that experimental hyperosmolality like exercise, counteracts quite effectively the response to adrenergic constrictor fibre activation in the resistance vessels but not in the capacitance vessels.

The extent to which graded experimental hyperosmolality interfered with the neurogenic constrictor response of the resistance vessels was investigated in some greater detail in another series of experiments (6 animals) performed under constant flow perfusion In these experiments, short hypertonic infusions were

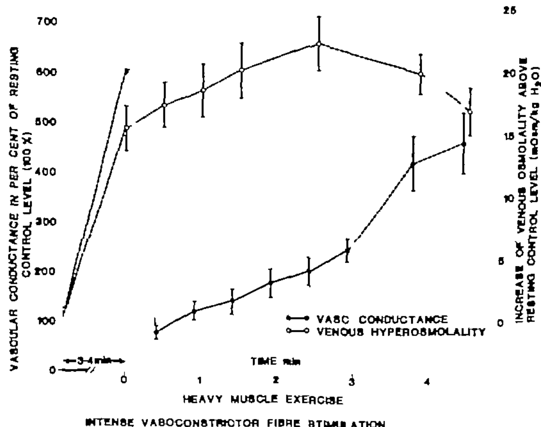


Fig 34 Diagram showing collected data on changes of vascular conductance and venous hyperosmolality during heavy exercise and superimposed intense sympathetic nerve stimulation. Each point represents mean ± SEM of 16 observations.

similar but the relative increase of venous osmolality during sympathetic stimulation was more pronounced (about 100 per cent increase)

The data taken together from the described three series of experiments indicate that tissue hyperosmolality interferes with the sympathetic constrictor influence on the resistance vessels during exercise and that a gradual enhancement of tissue hyperosmolality of exercise *per se* during sympathetic activation may reinforce this inhibitory action. The flow reduction caused by sympathetic stimulation seems mainly responsible for the gradual increase of osmolality during exercise, since mechanical flow reduction during work was found also to lead to a similar osmolar increase. Apparently the neurogenic flow reduction interferes with tissue nutrition, as indicated by the concomitant decrease of muscle force and may in turn cause an increased anaerobic metabolism and, hence, an increased osmolality for instance by greater lactate production The increased osmolality may be due to a reduced osmolar wash-out from th[illegible] blood flow decreases. To

some, but, on average probably only to a relatively small extent the observed increase of venous osmolality may reflect an improved transcapillary osmolar extraction (*cf* Chapter IV) and thus not a true increase of interstitial osmolality

Hyperosmolar versus myogenic reactions The reactions of the resistance vessels and of the precapillary "sphincters were studied in the muscle vascular bed in response to increased transmural pressure (5 animals) The arterio-venous pressure gradient was kept constant and transmural pressure was varied by 25 mm Hg by equal shifts of both arterial inflow and venous outflow pressures. High transmural pressure did not cause any augmentation of blood flow in the resting control state (autoregulation in the resistance vessels) but decreased the capillary filtration coefficient (CFC), on the average by 33 per cent indicating a myogenic constriction of the precapillary sphincters (10 observations) During hypertonic infusion, which raised venous osmolality by some 30 mOsm high transmural pressure increased blood flow above the level at low transmural pressure indicating impaired autoregulation in the resistance vessels, whereas CFC still decreased by an average of 38 per cent (16 observations) Papaverine infusion superimposed on the experimental hyperosmolality eliminated the latter effect indicating that the described decrease of CFC was due to an active response of the precapillary "sphincters" It thus appears that the dilator action of experimental hyperosmolality can override the myogenic response in the resistance vessels (when examined as the overall resistance function) whereas the myogenic constrictor response of the smallest precapillary vessels (precapillary "sphincters") is still maintained.

COMMENTS

The results presented indicate that during experimental hyperosmolality the vascular response patterns to the adrenergic and myogenic control systems are modified in much the same way as is known to occur during exercise (Kjellmer 1965 *a*, Lundvall *et al* 1967) These findings lend support to the view that tissue hyperosmolality is a mediator of exercise hyperemia. The efficiency of experimental hyperosmolality of magnitudes similar to those encountered in exercise, in counteracting the resistance effect to adrenergic vasoconstrictor fibre activation (Figs. 31 and 32) may suggest that the corresponding counteracting effect of exercise itself in fact, can to a significant extent be related to the work induced tissue hyperosmolality The finding that sympathetic vasoconstrictor fibre activation superimposed on exercise caused a clear-cut increase of tissue osmolality in excess of that produced by work alone (Figs. 33 and 34) and that this osmolar change parallelled the gradual decline of the constrictor effect, strengthens this suggestion. It should be pointed out, however that these observations were obtained during such an intense vasoconstrictor fibre activation that it led to a relatively well maintained blood flow impedement. Experiments using more physiological stimulation rates and different work intensities might reveal whether the phenomenon of adrenergic reinforcement of hyperosmolality can be of significance during normal exercise in the intact organism.

It should be stressed that other metabolic factors than hyperosmolality in all probability can counteract the sympathetic constrictor influence of the resistance vessels during exercise. Thus it has been shown that sympathetic vasoconstriction in resting muscle is much lessened or even abolished during hypoxia and during perfusion with blood with various combinations of hypoxia, increased potassium concentration and hyperosmolality (Skinner and Costin 1969 1970 1971) These three factors which all seem involved in the exercise hyperemia response, were shown to counteract the sympathetic constriction synergistically

CHAPTER VI

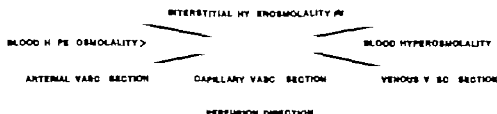
AN EVALUATION OF POSSIBLE TRANSINTIMAL DILATOR EFFECTS DURING HYPERTONIC INFUSION

A model study on the vascular bed of the intestine

The role of hyperosmolality in exercise hyperemia was evaluated in Chapter II by comparing vascular conductance changes and venous osmolar changes during exercise and during "experimental hyperosmolality the latter produced by short close i.a. infusions of hypertonic solutions to resting muscle. It was thus assumed that not only during exercise but also during hypertonic infusion vascular smooth muscle was affected by the hyperosmolality created in the interstitium and reflected in the venous effluent. To be able to produce the same rapid interstitial osmolar changes by hypertonic infusion as observed during exercise, the osmolality of the arterial blood had to be raised to relatively high levels. Since the vascular effects were evaluated in relation to venous and not to arterial hyperosmolality the latter being more pronounced it follows that the importance of tissue hyperosmolality in exercise hyperemia might have been over estimated if there were a significant direct transintimal dilator effect on the smooth muscle from the intraluminal side of the arterial vessels.

The question whether the dilator effect of hypertonic infusion is exerted from the interstitium after transcapillary osmolar exchange or if there is a significant transintimal influence as well, seems very difficult to approach in a "truly" physiological preparation. Some information of relevance to this problem may however be obtained from the present "model experiments although admittedly they deviate from normal physiological conditions.

The experimental approach implied that dilator effects of the resistance vessels to hypertonic infusion were compared during perfusion of a vascular bed in the normal direction from artery to vein (below denoted "forward perfusion") and during perfusion in the reverse direction from vein to artery ("retrograde perfusion") Such experiments can be performed on the small intestine, because its vascular bed has no or only rudimentary venous valves (*cf* Alexander 1963). As will be shown this vascular bed like that in skeletal muscle, dilates in response to hyperosmolality The idea behind this approach may be outlined with reference to the schematic drawing in Fig 35 During forward perfusion hypertonic infusion into the feeding (arterial) vessel produces an increased interstitial osmolality the extent of which is reflected in the effluent from the vein concomitantly the arterial blood osmolality exceeds significantly the interstitial osmolality During retrograde perfusion the hypertonic infusion into the feeding



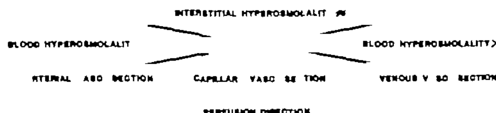


Fig 35 Relations between osmolality levels in inflow and outflow blood and in interstitium during forward" and retrograd perfusion of the intestine in hypertonic infusion experiments.

(venous) vessel can be adjusted so as to cause the same degree of interstitial hyperosmolality now reflected in the effluent on the arterial side concomitantly the osmolality of the blood passing the venous section is higher Interstitial osmolality can thus be raised to similar levels in both types of perfusion but the intraluminal osmolality in the arterial vessels, the main place of resistance in both experiments (see below) will be considerably greater during forward than retrograde perfusion. It follows that at comparable increases of the osmolality in the outflows, any significant transintimal osmolar resistance effect would be revealed as a greater dilator response during forward than retrograde perfusion if on the other hand similar dilator effects were elicited, hypertonic infusion would seem to exert its vascular effect mainly from the interstitial space after transcapillary osmolar exchange

METHODS

Experiments were performed on 17 cats of both sexes weighing 2.6 — 4.5 kg and anaesthetized as described in Chapter II Body temperature was maintained at 37,5—38,5 C using a heating pad and a heating lamp

The preparation used was basically that described by Folkow *et al.* (1963). The abdomen was opened in the midline and the greater omentum and the spleen extirpated. A section of the jejunum-ileum, weighing 15 — 55 g, was used for the study the remaining part of the intestinal tract being removed. Care was taken to leave the superior mesenteric artery and vein with their vascular

branches to the intestinal segment intact. The vascular bed of the segment was denervated by cutting the nerve plexuses surrounding the superior mesenteric artery After heparinization (3 — 5 mg/kg b w) a siliconized shunt tubing was inserted which diverted flow from the right femoral artery to the intestine *via* the cannulated superior mesenteric artery The superior mesenteric vein was cannulated and the venous outflow from the region was measured continuously by diverting flow through an optical droprecorder unit after which flow was returned to the animal *via* a funnel connected to the right jugular vein Venous outflow pressure was determined by the height above the preparation of the ori fice of the outflow tubing. The hypertonic infusions were given proximal to a specially designed "mixing chamber" inserted in the inflow tubing to ensure thorough mixing with blood (see Chapter II) Inflow blood pressure was moni tored from a T-tube close to the feeding vessel Retrograde perfusion was ac complished simply by shifting the arterial and venous tubings at the cannulation sites of the mesenteric artery and vein As will be described, retrograde perfusion was only possible if inflow pressure was reduced and this reduction was achieved by a screw-clamp placed around the abdominal aorta.

In some experiments the intestinal segment was enclosed in a perspex plethysmograph filled with Tyrode's solution (38 C) to allow continuous recording of changes in tissue volume and determinations of the capillary filtration coeffi cient (CFC) see Folkow *et al* 1963

Isotonic and hypertonic (≈1700 mOsm/kg H_2O) glucose (or sometimes suc rose or NaCl) solutions were infused at rates between 0.2 — 3 4 ml/min using a constant infusion apparatus. Plasma osmolality was determined by cryoscopy (Chapter II) in blood samples withdrawn from T-tubes in the shunt circuit distal to the mixing chamber (arterial blood) and in the outflow tubing (effluent blood) After the osmolality determination, the sampled blood was returned to the animal to minimize the blood loss.

Isoprenaline, used as a reference substance to induce a maximal resistance ves sel dilatation, was infused in a dose of 0 4 — 0 8 µg/min (*cf* Folkow *et al.* 1963) Mechanical interference with blood flow from intestinal smooth muscle activity was avoided by administration of atropine (1 mg/kg b.w) in the beginning of the experiment. This also minimized secretory activity Tissue weight was deter mined for a segment of small intestine of equal length to that under study

RESULTS

Three different types of experiment were performed and the results will be presented in separate sections. In the first section (*A*) the reactions of the intestinal resistance vessels to graded hypertonic infusion under "normal" experimental conditions will be described. The second section, (*B*), describes conditions under which retrograde perfusion can be performed and, further the resistance distri

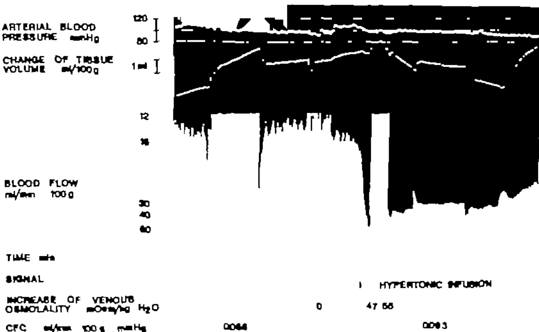
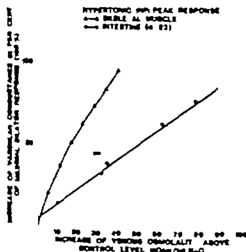


Fig 36 Changes of blood flow and CFC produced in the small intestine by intra-arterial hypertonic infusion. Flow increased rapidly to a peak value and then decreased during continued infusion to ttain an approximately steady state level of hyperemia. Changes of venous osmolality and of CFC indicated t bottom of the figure.

bution between the arterial and the venous side of the vascular bed during forward and retrograde perfusion. Finally in the third section, (C), the problem whether hyperosmolality during hypertonic infusion influences vascular smooth muscle from the interstitial space or directly from the blood stream was approached.

A Fig 36 shows the typical pattern of blood flow changes to hypertonic infusion observed during perfusion in the normal direction and at a normal pressure head. It can be seen that blood flow which was about 14 ml/min x 100 g tissue in the control period, increased rapidly after the onset of the hypertonic infusion (glucose solution) to reach a maximal value of about 60 ml/min x 100 g within 30 sec ("peak response") after that it decreased somewhat to attain an approximately steady hyperemic level of about 40 ml/min x 100 g ("steady state response") during continued infusion. At the peak response venous osmolality reflecting interstitial osmolality was raised by 47 mOsm/kg H_2O and in the early steady state period by 55 mOsm above the control level. Upon cessation of the infusion blood flow gradually declined towards the control level. The pattern of response of the resistance vessels to hypertonic infusion is thus similar to that observed in skeletal muscle (*e g* Figs. 5 and 6, Chapter II) The increased CFC during hypertonic infusion (also noted in the other plethysmo-



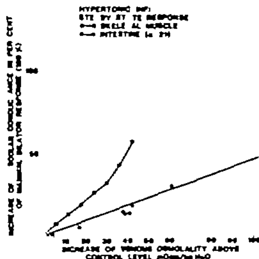


Fig 37 Diagram showing "peak and steady state" dilator responses of the intestinal resistance vessels during i. a. hypertonic infusion against the concomitant increase of venous osmolality (linear regression lines drawn). Osmolar conductance effects expressed in per cent of the maximal conductance increase possible to evoke by i. a. infusion of supramaximal dose of isoprenaline. Data refer to experiments in which the intestine was perfused at a normal pressure head. For comparison corresponding osmolar effects evoked in skeletal muscle are shown.

graphic experiments) suggests a concomitant dilatation of the precapillary sphincters as in skeletal muscle (*cf* Chapter II) (It should be mentioned that the CFC determination during infusion could be made under constant pressure perfusion in the intestine which proved difficult in skeletal muscle (Chapter II) This discrepancy seemed related to the fact that osmotic transcapillary fluid absorption was found to be less pronounced in the former than in the latter tissue)

The dilator effects of the intestinal resistance vessels to graded experimental venous hyperosmolality determined during the peak response and in the early phase of the steady state response (*cf* Fig. 36), were investigated in 6 animals. In each preparation, repetitive infusions were given at intervals permitting full recovery of vascular tone in between the infusions. In the individual animal there was a relation between the magnitude of the dilator response and the degree of hyperosmolality both with regard to the peak and the steady state response.

Considerable variation of the vascular tone in the control state in preparations from different animals (vascular conductance 0 13 — 0 67 mean value 0.31 (ml/min x 100 g)/mm Hg) made it difficult to make a quantitative evaluation on the whole material since as discussed in Chapter II the magnitude of a dilator response is influenced by the control tone. The material in the present

study was too small to compensate adequately for this variation in the way described in Chapter II In an attempt to circumvent this difficulty the hyperosmolar dilator effects (vascular conductance during hypertonic infusion minus control vascular conductance prior to the infusion) were expressed in per cent of the maximal dilator response (vascular conductance at maximal vasodilatation (0 96 ± 0 133 SEM (ml/min x 100 g)/mm Hg) minus control vascular conductance prior to the hypertonic infusion) evoked repetitively in each animal by a short intra arterial infusion of a supramaximal dose of isoprenaline (Fig. 37) It can be seen that the dilatations elicited both at the peak and at the steady state response were correlated to the degree of venous hyperosmolality (r = 0.82, p < 0 001 and r = 0 81 p < 0 001 respectively) Both the peak and the steady state vascular responses seem roughly linearly related to the increase of venous osmolality as depicted by the drawn regression lines. For comparison, corresponding data for skeletal muscle, recalculated from Fig. 9 in Chapter II are shown (broken lines) As a measure of maximal dilatation in skeletal muscle the data during heavy work were used. It is clear that at comparable levels of venous hyperosmolality the dilatations in skeletal muscle are significantly more pronounced than those in the intestine both with regard to the peak and to the steady state response. Nevertheless, since the intestinal dilator effects are clear-cut and correlated to the induced hyperosmolality a basic prerequisite for use of the intestine to approach the problem of possible transintimal influence of hypertonic infusion on the vascular smooth muscle is fulfilled

B Pilot experiments showed that the intestinal vascular bed could be perfused for a considerable length of time in the retrograde direction provided inflow pressure was reduced down to the range of 40 — 50 mm Hg. At a normal pressure head, on the other hand, flow virtually stopped after a few minutes, an observation in accordance with that of Alexander (1963) Fig. 38 shows a typical flow pattern during forward perfusion (Panel A) and during retrograde perfusion (Panel B) at the same pressure head (inflow pressure approximately 45 mm Hg and outflow pressure 2 mm Hg) It can be seen that in the initial period during retrograde perfusion blood flow was only moderately reduced compared to the level at forward perfusion. There was, however some gradual decline with time, probably due to the progressive oedema formation evidenced by the increase of tissue volume. The last part of Panel B shows that blood flow almost stopped when perfusion pressure was raised to about 110 mm Hg (at the arrow the rising rate for the blood flow ordinate writer was reduced blood flow was thus in fact the same as just before the arrow) This effect could tentatively be ascribed to a myogenic constrictor response, a critical rise of tissue pressure seems to be a less likely explanation since, after an initial capacitance response, the fluid filtration rate was about similar to that during the low pressure head (see volume tracing)

A prerequisite for the study in section C below is that the resistance distribution between arterial and venous vessels is similar during forward and retro-

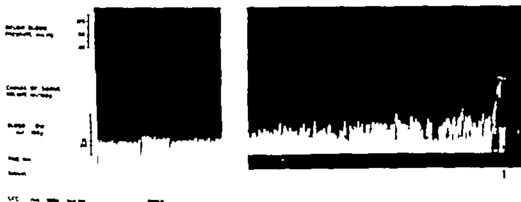


Fig 38 Intestinal blood flow and tissue volume during forward perfusion (panel A) and retrograde" perfusion (panel B). Retrograde perfusion only possible during reduced inflow pressure. For further explanation see text.

grade perfusion A deduction of the pre-/postcapillary resistance ratio can be performed with the aid of the volume curve and the CFC determination (regardless of perfusion direction precapillary resistance will below refer to the arterial and postcapillary to the venous resistance) In the isovolumetric state during forward perfusion at a normal pressure head (about 100 mm Hg) mean hydrostatic capillary pressure (P) in the intestine has been estimated to be about 15 mm Hg (Folkow *et al* 1963) a value adopted for the present deductions. Under these circumstances the pre-/postcapillary resistance ratio is about 6:1 When inflow pressure was reduced an approximate isovolumetric state was still present (see Fig. 38 Panel A) indicating that P was maintained at about 15 mm Hg. This Pc value gives a pre-/postcapillary resistance ratio of 2.3 1 (inflow pressure 45 mm Hg and outflow pressure 2 mm Hg), which in turn permits the determination of the CFC which was 0 089 ml/min x 100 g x mm Hg. Since the slight tissue volume decline in Panel A (0 16 ml/min x 100 g) indicated some deviation from a true Starling equilibrium, a more correct value for P may be 13 mm Hg (15 mm Hg —(0 16/0 089) mm Hg) this gives a corrected value for pre-/postcapillary resistance of 2 9:1 (note that the CFC value is hardly affected by such a small correction of the resistance ratio) Reduction of perfusion pressure during forward perfusion which led to some decrease of overall resistance, was thus associated with a decline of the pre-/postcapillary resistance ratio, apparently due to efficient autoregulatory adjustments mainly in the precapillary resistance vessels. The major resistance is, however still confined to the last-mentioned vascular section

The observed filtration rate in the early (<5 min) phase of retrograde perfusion indicated that P had risen by about 20 mm Hg compared to the preceding forward perfusion, assuming that CFC was the same as before. Thus P would be 33 mm Hg giving a pre-/postcapillary resistance ratio of 2.6 1 The

resistance distribution along the vascular bed during normal and retrograde per fusion at reduced inflow pressure therefore apparently is quite similar a conclusion reached also in the other experiments of this type. Mention may be made that CFC during retrograde perfusion possibly could have been somewhat lower than assumed due to myogenic constrictor mechanisms. If so, the resistance ratio would have been even higher The important thing for the analysis in section C is, however that the pre-/postcapillary resistance ratio during retrograde per fusion is not significantly *lower* than during normal perfusion.

C In this section a quantitative comparison of the resistance vessel response to hypertonic infusion during forward and during retrograde perfusion was made (8 animals) both types of perfusion being performed at reduced inflow pressure (40 — 55 mm Hg) In the same animal, periods of forward perfusion alternated with relatively short periods of retrograde perfusion The marked oedema formation occurring during prolonged retrograde perfusion (Fig 38) was thereby minimized. The hypertonic infusions also helped to limit the oedema formation as a result of the evoked osmotic fluid absorption

The level of vascular tone present in the intestine during forward and retrograde perfusion could be determined by administering a supramaximal dose of isoprenaline. The control vascular conductance before hypertonic infusion averaged 0.35 ± 0 030 SEM (ml/min x 100 g)/mm Hg during forward perfusion (n=25) and 0.23 ± 0.028 SEM (ml/min x 100 g)/mm Hg during retrograde perfusion (n=19) Vascular conductance during forward perfusion was thus 52 per cent higher than during retrograde perfusion Vascular conductance during isoprenaline infusion averaged 0.74±0 062 SEM (ml/min x 100 g)/mm Hg during forward perfusion and 0 48 ± 0 050 SEM (ml/min x 100 g)/mm Hg during retrograde perfusion, *i.e* the difference during maximal dilation was in fact almost exactly the same (54 %) as in the control situation The lower vascular conductance during retrograde perfusion than during normal perfusion both in the control state and at maximal dilatation apparently must be ascribed to some physical factor(s) Note that, despite this difference, the relative vascular conductance increase from the control level in response to isoprenaline was equal (≈110 %) during both types of perfusion indicating that the level of "active' vascular tone in the control state was of comparable magnitude in both cases.

Hypertonic infusion was found to elicit a clear-cut resistance vessel dilatation both during forward and retrograde perfusion, as illustrated in Fig 39 *During normal perfusion (Panel A) a venous osmolality increase* by 65 mOsm was associated with an initial blood flow increase from about 11 ml to about 20 ml/min x 100 g tissue after which flow declined somewhat (steady state response) during the continued hypertonic infusion. Upon cessation of the infusion blood flow gradually returned to the control level A later administered supramaximal dose (5 μg) of isoprenaline raised blood flow to about 23 ml/min x 100 g tissue at the same pressure head This response could be considered to represent a ma

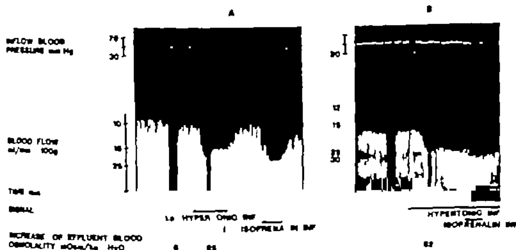


Fig. 39 Resistance vessel dilatations evoked by hypertonic infusion during forward (panel A) and retrograde (panel B) perfusion of the intestine. Observed increases of osmolality i the effluent blood indicated. Maximal vasodilatations evoked by isoprenaline.

ximal resistance vasodilatation (see above) The peak dilator response to hypertonic infusion thus corresponded to about 75 per cent of the maximal dilator response (calculated as described for Fig 37) During retrograde perfusion (Panel B) an increase of the osmolality in the effluent by 52 mOsm was associated with an initial blood flow increase from about 15 ml to about 25 ml/min x 100 g after which there was some decrease of the hyperemia This dilator response was 60 per cent of the maximal dilator response evoked by the superimposed isoprenaline infusion taking the slight but significant pressure changes into account. The magnitude of the hyperosmolar dilator responses during the two types of perfusion are not very different in view of the somewhat different osmolalities produced (*cf* also Fig. 37) Mention may be made that during reduced inflow blood pressure the peak to steady state pattern of flow response was in general clearly less pronounced than in the experiments described in section *A* above in which the intestine was perfused in the normal direction at a normal pressure head (*cf* Chapter II, A2)

The collected data from this series of experiments are shown in the diagram of Fig 40 in which the dilator effects to hypertonic infusion during forward (closed circles, n = 25) and during retrograde (open circles, n = 13) perfusion are expressed (as above) in per cent of the maximal dilator effect evoked by isoprenaline. The responses are plotted against the induced hyperosmolality of the effluent blood. Correction was made for any rheological effect of the

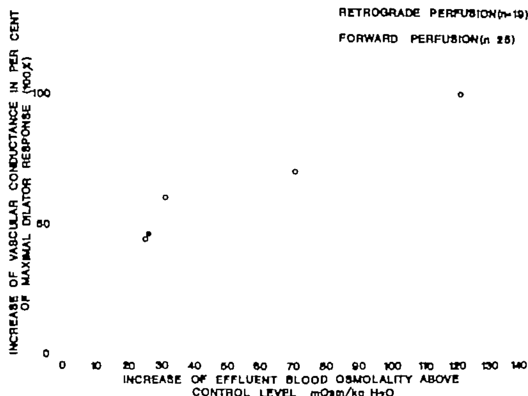


Fig 40 Diagram showing intestinal resistance eased dilator responses to hypertonic infusion during forward and retrograde perfusion against concomitantly observed increases of effluent plasma osmolality Dilator effects expressed in per cent of maximal dilator response evoked by isoprenaline.

fused fluid *per se* such effects were revealed by routine isotonic control infusion The data refer to observations in the early hyperemic phase during the hypertonic infusion because a possible transintimal dilator influence on the vascular smooth muscle should be best revealed at the time when the difference between the hyperosmolality of the perfusate on the inflow side and of the interstitial fluid (≈outflow hyperosmolality) was most pronounced (*cf* description to Fig. 35) This osmolar difference of course gradually declined with time both during forward and retrograde perfusion. Both during forward and retrograde perfusion there was a correlation between the dilator response and the evoked hyperosmolality of the effluent blood ($r = 0.60$ $p<0.01$ and $r = 0.74$ $p<0.001$ respectively) There was no significant difference between the dilator responses during forward and during retrograde perfusion at any of the hyperosmolality levels produced as shown by linear regression analysis (Scheffe s method, Miller 1966 *cf* Chapter II, A1)

The osmolality of the perfusate on the inflow side was determined during the development of the dilator response to hypertonic infusion and compared to

that on the outflow side determined during the phase when the dilator response was fully developed during forward (n = 8) as well as during retrograde (n = 8) perfusion. On the average the inflow hyperosmolality exceeded the outflow hyperosmolality by a factor of 4.1 during forward perfusion and by a factor of 4.3 during retrograde perfusion. Since the main vascular resistance is located in the arterial section both during forward and during retrograde perfusion (Fig. 38), these pronounced differences between inflow and outflow hyperosmolality should have caused much greater dilator responses during forward perfusion than retrograde perfusion at comparable levels of effluent hyperosmolality if any significant transintimal osmolar dilator effect had been present. However no such difference between the dilator effects was found (Fig. 40), a fact which indicates that the dilator effect on the resistance vessels to short term hypertonic infusion is mainly exerted from the interstitial space after transcapillary osmolar exchange.

COMMENTS

Blood-borne vasoactive agents, occurring either naturally in the intact organism or administered experimentally can gain excess to the vascular smooth muscle cells from the interstitial space of the organ after transcapillary exchange or directly from the blood stream *via* penetration of the intima and associated structures of the vascular wall ("transintimal effect") In larger conduit vessels, the existence of vasa vasorum provides another possibility but in smaller species (adult b.w <5—6 kg) like the cat, no vasa vasorum seem present, at least not in the small arteries (*cf* Somlyo and Somlyo 1968). Little seems to be known about which of the routes to the target cells, the transcapillary or the transintimal, is the most important one and, apparently it may differ with regard to the characteristics of the vasoactive agent in terms of its ability to penetrate membrane barriers, as determined, for instance, by molecular size and degree of lipid-solubility The problem may become of significance in experimental work. For example, studies of local regulation of blood flow like the search for the factor(s) responsible for functional hyperemia in skeletal muscle, usually involve intra-arterial administration of vasoactive agents. The question then arises whether the evoked vascular effects should be related to the induced interstitial (venous) concentration of the particular substance, as is usually done, or to the arterial blood concentration. Since the latter generally exceeds the venous concentration, at least in short-term infusion experiments, this question is an important one.

The present study was performed in an attempt to investigate this problem with regard to hyperosmolality produced by intra-arterial hypertonic infusion It was prompted by the fact that, if a significant transintimal dilator effect were evoked, the data presented in Chapter II would have overestimated the role of tissue hyperosmolality in the exercise hyperemia response as explained in the introductory remarks above. Since the problem seemed very difficult to app-

roach under "truly" physiological conditions, special model" experiments were performed. Admittedly the experimental approach involved a major unphysiological procedure, *i.e.* "retrograde perfusion" of the intestinal vascular bed. However by performing this type of perfusion at a reduced inflow pressure and for only relatively short periods of time, which among other things limited marked oedema formation, the vasculature during retrograde perfusion was found to exhibit, in essential respects, similar characteristics to those shown during perfusion in the forward direction at similar levels of inflow pressure. Thus the control state active vascular tone, the pre-/postcapillary resistance ratio as well as the maximal dilator responses which could be evoked were comparable during the two types of perfusion.

The results presented seem strongly to suggest that the vascular dilator effect of short term hypertonic infusion is mainly exerted from the interstitial space *after transcapillary osmolar exchange and that transintimal influence on the vascular smooth muscle* is of little importance. The present study therefore supports the validity of the evaluation of the role of regional tissue hyperosmolality for the functional hyperemia in skeletal muscle as depicted by Fig 14 in Chapter II. The typical resistance response in muscle to hypertonic infusion in terms of a "peak" flow increase followed by a less pronounced "steady state" hyperemic phase (Chapter II) could *a priori* be ascribed to a significant transintimal dilator effect early during the infusion. This because there will initially be a higher intraluminal hyperosmolality on the arterial side before blood flow increases than after *i.e.* before the dilution of the infused solution is increased. However this interpretation thus seems denied by the present data. This seems to remove one important objection against using the peak flow response to hypertonic infusion in the quantitative evaluation of the causal role of tissue hyperosmolality in exercise hyperemia (Fig. 14 Chapter II)

Attempts to explain the present findings must necessarily be speculative. The *dilator effect of hyperosmolality seems related to an osmotic shrinkage of the* vascular smooth muscle cells as shown by several *in vitro* studies (see General Discussion) Such an effect could be brought about *via* transintimal solute transfer from the blood into the interstitium of the vessel wall and/or *via* transintimal attraction of fluid (water) from the wall It would appear that at *least* fluid passage could occur and that therefore some transintimal influence might be present. With regard to the *relative* importance of transintimal effects on the one hand and effects exerted after transcapillary osmolar exchange on the other the permeability characteristics of the intima and of the capillary membranes should be compared. The total capillary surface area no doubt is much greater than the total intimal surface area of the main resistance vessels (small arteries and arterioles) and the permeability per unit surface area, in addition, is probably significantly greater for the capillaries than for the arteriolar intima. It would seem therefore that the capillaries may provide a much faster route for osmolar exchange with the immediate environment of the vascular sm[illegible] cells

and thereby explain the present findings. Some transintimal dilator effect how ever can of course not be excluded, especially not during prolonged hypertonic infusion.

Two recent reports deserve consideration in this connection Duling and Berne (1970) obtained evidence indicating that oxygen easily diffuses across the vas cular wall of precapillary vessels. This is not surprising in view of the fact that oxygen is highly lipid-soluble and therefore penetrates membranes much more easily than the lipid-insoluble substances used in the present study More diffi- cult to explain in view of the present results is the finding of Uchida *et al* (1967) that the perfusion of isolated small precapillary vessels (o.d. 250—50μ) with solutions containing vasoactive agents, such as epinephrine norepinephrine se- rotonin, angiotensin and potassium chloride, evoked clear-cut constrictor effects. It appears that these effects were exerted from the luminal side. It might be ar gued, however that the transintimal permeability of these isolated vessels, which often were stored for several days before the experiments, could possibly have been abnormally high thus fascilitating transintimal exchange. *In vivo* humoral control of the peripheral circulation might be exerted mainly after transcapil lary substance exchange as suggested by the present study

CHAPTER VII

GENERAL DISCUSSION

Skeletal muscle tissue exhibits a very rapid and pronounced increase of its metabolic rate when work begins. That the raised metabolism leads to an increased number of osmotically active particles in the tissue was suggested a long time ago (e.g. Barcroft and Kato 1915 cf Landis 1934) It has also been known for some time that hypertonic infusions can evoke decreased resistance in the peripheral circulation (e g Marshall and Shepherd 1959 Overbeck *et al* 1961 Fröhlich 1966) However a direct physiological role of tissue hyperosmolality in vascular control was suggested only recently when experimental evidence was presented to indicate that tissue hyperosmolality was causally linked to the exercise hyperemia response (Mellander *et al* 1967).

The present study is an extension of our previous work; by detailed analyses of the vascular effects in muscle during hyperosmolality induced by exercise and by hypertonic infusion in resting tissue a quantitative evaluation of the role of tissue hyperosmolality in exercise hyperemia was performed. Another aim was to elucidate the mechanisms responsible for the considerable transcapillary fluid movement that occurs in muscle during and after work, special attention being paid to possible osmotic processes. The first mentioned problem was investigated by different experimental approaches reported in Chapter II III V and VI and the latter problem was dealt with in Chapter IV

Most of the present experimental findings have already been commented upon in the previous chapters, where the results were subjected to a critical evaluation. This part of the paper will therefore only present a short survey and discussion of the main results and conclusions together with some integrative aspects of the exercise hyperemia response and of the transcapillary fluid dynamics during work.

In Chapter II it was shown that muscle exercise was associated with a considerable regional tissue hyperosmolality the extent of which was estimated in the venous effluent from active muscle (e g Fig 2) The hyperosmolality developed almost immediately at the commencement of work and within a few minutes it reached a maximal level. The dilator response of the resistance vessels showed a similar time-course. The extent of venous hyperosmolality during short term (<5 min) exercise was correlated to the magnitude of the concomitant dilator response of the resistance vessels, both these phenomena, in turn, being correlated to the "intensity (somato-motor fibre stimulation rate, 0.25—4 imp/sec) of the work (e g Fig 4) During "heavy (4 imp/sec) exercise venous osmolality could exceed that at rest by as much as 40—45 mOsm/kg H_2O During

more prolonged work, up to 15 min duration, venous hyperosmolality remained at the peak level reached within the first few minutes when exercise was "light" (<1 imp/sec) or "moderate" (1—2 imp/sec) but tended to decline during prolonged "heavy" work a decline which then ran parallel to a decline of the developed muscle force (Table I) — The bulk of data were obtained during short term exercise and the main emphasis will be placed on these results.

Analysis of the constituents of the venous effluent from exercising muscle indicated that the hyperosmolality could be ascribed mainly to increased concentrations of sodium and lactate, but also of some other substances, such as potassium (Table II) Release of substances into the interstitial space from the contracting striated muscle fibres and osmotic fluid (water) movement into these cells can explain the changed composition of the extracellular fluid.

In order to analyse in quantitative terms the effects of hyperosmolality *per se* on the resistance function, an "experimental tissue hyperosmolality" was produced in resting muscle by close arterial hypertonic infusion To be relevant to the above-mentioned problem of exercise hyperemia it was considered important to design these experiments so that the osmolar changes produced in the tissue (again estimated in the venous effluent) resembled those of exercise not only with regard to *magnitude* but also to *time-course* Experimental hyperosmolality was always associated with a rapidly developing dilator response of the resistance vessels giving an initial peak blood flow increase ("peak response") followed by a somewhat less pronounced steady hyperemia level ("steady state response") indicating a secondary partial recovery of vascular tone during continued infusion (e.g Figs. 5 and 6) Observations were also obtained during constant flow perfusion but under these circumstances such partial recovery of vascular tone was not observed (Fig 8) There was a correlation between the magnitude of the dilator response of the resistance vessels and the concomitant increase of venous hyperosmolality both with regard to the peak and steady state response observed during "constant pressure perfusion and with regard to the resistance decline during constant flow perfusion (Figs 9—11) At the a virtually maximal resistance vessel dilatation was present venous osmolality by 40—50 mOsm whereas to maintain a as steady state response, venous osmolality had to be raised by

A critical analysis of the observed effects on the infusion strongly suggested that after due corr infused fluid *per se* and of the induced transcap they could be considered to be caused by d muscle tone on an osmotic basis As will be d strongly supported by *in vitro* studies on vascular effect on the vascular smooth muscle of hypertoni the interstitial space after transcapillary osmc significant extent from the luminal side of the mal" influence. The latter possibility seemed

Chapter VI This analysis was prompted by the fact that in the infusion experiments the hyperosmolality in the arterial blood exceeded that of the venous blood, especially in early phases of infusion In Chapter II the vascular effects of hypertonic infusion were, however evaluated in relation to the induced venous hyperosmolality

In this connection the pattern of vascular response in terms of a peak response and a later steady state response to hypertonic infusion deserves consideration. Had a significant transintimal osmotic effect been present it could have explained the early pronounced dilatation, but this possibility seemed excluded by the results of Chapter VI. A more likely explanation for the partial recovery of vascular tone during hypertonic infusion (steady state response) was, however offered by some results described in Chapter III It was shown here that a clear-cut rise of pressure seemed to develop in the micro-vessels just prior to the transition of the peak to the steady state response (Fig. 18) The pressure increase became pronounced and was maintained during the steady state response but such an effect was not present during exercise nor during the early phase of the peak response. The initial pressure rise could at least partly be ascribed to trans capillary absorption of fluid to the blood stream during hypertonic infusion and a probable decrease of interstitial pressure consequent to the fluid transfer would make the transmural pressure increase quite pronounced. A transmural pressure rise caused by the osmotic fluid absorption can be considered an experimental artefact"

Circumstantial evidence (Chapter II and III) suggested that the described transmural pressure rise evoked a secondary myogenic constrictor response in the small precapillary vessels during hypertonic infusion which counteracted the true dilator effect of hypertonicity It follows that the peak dilator response may be considered more relevant than the steady state response when evaluating the quantitative role of tissue hyperosmolality in exercise hyperemia. This conclusion was supported by the fact that the dilator effects during the "peak response" were quantitatively very similar to the effects obtained during hypertonic infusion under constant flow perfusion Under constant flow a myogenic response might well not develop since the perfusion pressure drop during dilatation should tend to balance an increase of transmural pressure.

A quantitative evaluation of the role of tissue hyperosmolality in short-term exercise hyperemia was performed by expressing the resistance vessel dilator response during experimental hyperosmolality in per cent of the exercise hyperemia response at corresponding levels of venous hyperosmolality (Fig. 14 Chapter II) When judged from the peak response during hypertonic infusion, or from the constant flow perfusion experiments, tissue hyperosmolality might explain some 30 to 90 per cent of the exercise hyperemia. When judged from the steady state response, on the other hand, the percentage was 15—60 For all three curves (Fig. 14) the percentage increased with increasing osmolality Since the work induced tissue hyperosmolality was correlated to the work intensity this suggests

that hyperosmolality is of greatest significance during heavy work. Irrespective of which set of data is used for the evaluation, tissue hyperosmolality can be considered an important mediator of exercise hyperemia. — If as suggested above, the peak and constant flow data are more relevant to the problem than the steady state effects, the role of hyperosmolality is indeed pronounced. When the peak response data and the constant flow perfusion data are considered together with *average* values for tissue (venous) hyperosmolality induced by exercise of different intensities, more than 40 per cent of the exercise hyperemia during light, and more than 60 per cent during heavy short term exercise can be ascribed to tissue hyperosmolality It should finally be mentioned that since a complete transcapillary osmolar equilibrium apparently was not established during exercise and during experimental hyperosmolality (Chapter II section A 1 and A 2, Chapter IV) the data given in Fig. 14 may represent a certain underestimation of the role of tissue hyperosmolality in exercise hyperemia.

Any metabolic factor which plays a causal role in exercise hyperemia should, upon administration to the resting muscle in amounts similar to those believed to be established during exercise, elicit the same specific and differentiated pattern of vascular response in all sections of the vascular bed as the exercise itself (*cf* Kjellmer 1965 *b* and *c*) That this important demand is fulfilled by tissue hyperosmolality is shown by the various types of experiment reported in Chapter II and III Thus both exercise and experimental hyperosmolality evoked pronounced dilatation not only in the resistance vessels but also in the precapillary sphincters on the other hand neither exercise nor experimental hyperosmolality caused any significant active dilatation of the capacitance vessels and apparently no change of capillary permeability (Chapter II) In Chapter III a detailed analysis of the resistance ("resistivity") changes in very delimited parts of the vascular tree was carried out. The pattern of resistivity change during exercise and during the peak response to experimental hyperosmolality was found to be roughly similar

During exercise the most prominent effect of superimposed sympathetic constrictor fibre activation is a maintained constriction of the capacitance vessels whereas the constrictor effect of the resistance vessels in quite transient, and the main effect of increased transmural pressure is a myogenic constriction of precapillary "sphincters" (Kjellmer 1965 *a*, Lundvall *et al* 1967) The fact that the adrenergic as well as the myogenic control systems were shown to elicit these differentiated patterns of response during experimental hyperosmolality supports the view that tissue hyperosmolality is involved in the exercise hyperemia response (Chapter V)

The osmolality hypothesis has also been tested in experiments on man (Lundvall *et al* 1969) During strenuous forearm exercise a considerable increase of regional venous osmolality was found which could amount to almost 30 mOsm/kg H_2O Infusion of hypertonic solutions into the brachial artery in amounts that raised regional venous osmolality by up to 20 mOsm evoked a clear-cut decrease

of forearm flow resistance, sometimes down to about 25 per cent of the control value. The latter results were essentially confirmed by Overbeck and Grega (1970) The data considered together strongly suggest that tissue hyperosmolality is a causal factor involved in the exercise hyperemia that develops in our own skeletal muscles during voluntary exercise.

Interstitial deposits of ^{133}Xe in resting human or cat skeletal muscle were found to be cleared at faster rates when the tracer was dissolved in hypertonic medium than when it was dissolved in isotonic medium indicating that a local microvascular dilator response was evoked when the hypertonicity as in exercise, primarily involved the extravascular space and was thus not produced *via* the blood stream (Lundvall *et al* 1969 Gray *et al* unpublished observations) The clearance rates were related to the extent of local experimental hypertonicity and could be as large as those observed with isotonic Xenon in exercising muscle.

As discussed the present *in vivo* study strongly indicated that the observed vascular effects of hypertonicity were caused by direct inhibition of vascular smooth muscle tone on an osmotic basis. The mechanisms responsible for the inhibitory action of hyperosmolality have been elucidated in extensive studies of the electrical and mechanical activity in isolated, spontaneously active vascular smooth muscle preparations (Mellander *et al* 1967 Johansson and Ljung 1967 Johansson and Jonsson 1968 Arvill *et al* 1969 Johansson 1969) In brief these studies showed that increased osmolality caused pronounced and sustained relaxation of vascular smooth muscle, mainly by inhibiting myogenic pacemaker activity This effect, in turn, could be ascribed to changes in transmembrane ionic concentration gradients and in membrane permeabilities to ions produced by osmotic reduction of smooth muscle cell volume. Pronounced hyperosmolality also interfered with intercellular propagation and with excitation-contraction coupling. Therefore, hyperosmolality can exert a negative "chronotropic" inotropic" and "dromotropic" action on vascular smooth muscle. Exercise leads to pronounced hyperosmolality in the interstitium, *i.e.* in the immediate environment of the vascular smooth muscle, by production and release of osmotically active agents from the contracting skeletal muscle fibres. It is likely that this environmental change leads to shrinkage of the vascular smooth muscle cells and to the consequent series of electrical and mechanical events that were shown to produce relaxation in the *in vitro* experiments.

So far the main emphasis has been placed on the vascular effects observed during short term exercise and short term hypertonic infusions. The data taken together strongly suggest that tissue hyperosmolality is of importance for the elicitation and the maintenance of the dilator response during short-term exercise. The following observations suggest that tissue hyperosmolality is of importance for the dilator response also during more prolonged exercise. The hyperosmolality *as mentioned above, was found to be maintained at a high level for a* considerable length of time during exercise, at least when it was not too strenuous (Chapter II A1) Further hypertonic infusions elicited dilator effects throughout

prolonged infusion periods, but the dilator response was then usually maintained at a relatively steady level despite a gradual rise of osmolality (Fig 7) A myogenic constrictor mechanism could possibly explain this phenomenon as discussed above. Other alternatives, however deserve consideration. Since the inhibitory action of hyperosmolality on vascular smooth muscle seems related to cell shrinkage, the maintenance of a dilator effect would depend upon the ability of the osmotically active substance(s) to penetrate the vascular smooth muscle cell membrane. *In vitro* thus, urea hyperosmolality caused no clearly detectable shrinkage of the vascular smooth muscle cells and only a transient inhibition of the spontaneous rhytmic activity whereas sucrose hyperosmolality caused a sustained and maintained reduction of cell volume and an inhibition of "vascular tone which also was sustained although most pronounced during the first minutes (Johansson and Jonsson 1968 Arvill *et al* 1969) These differences were explained by the fact that urea, in contrast to sucrose, showed a rapid entrance into the cells. Among the substances tested in the present *in vivo* hypertonic infusion experiments (sucrose, glucose, mannitol, xylose, NaCl, sodium lactate, and urea) urea was the only that showed signs of fairly rapid penetration in terms of relatively transient dilator response (Chapter II A2) Since work induced interstitial hyperosmolality to a great extent seems related to increased concentrations of sodium and lactate (Table II) this may imply that vascular smooth muscle shrinkage during exercise is maintained for relatively long periods of time. Despite a maintained smooth muscle cell volume reduction it is possible, however that there is some recovery of vascular tone as suggested by the described *in vitro* experiments with sucrose it can not be excluded that such a phenomenon, if present *in vivo* to some extent could be responsible for the transition from the peak to the steady state response in the earlier phase of hypertonic infusion. Although no definite quantitative evaluation of the role of tissue hyperosmolality during more prolonged exercise can be given at present it is quite clear however that the rapid and pronounced changes of tissue hyperosmolality that occur during *short term* exercise will cause considerable inhibition of vascular smooth muscle tone and, hence, marked vasodilatation as summarized in Fig. 14

The hypothesis that tissue hyperosmolality is a mediator of exercise hyperemia has, besides in the studies discussed above, been approached also in some other laboratories. Thus, Scott *et al* (1970) and Scott and Radawski (1971) confirmed on dog skeletal muscle that exercise was associated with increases of regional tissue osmolality which, under conditions of constant flow perfusion, were of similar magnitudes to those reported in the present study and roughly maintained for the period of work. During constant pressure perfusion, however they found a decline of the work induced tissue hyperosmolality with time. They also studied vascular effects in resting muscle in response to prolonged hypertonic infusions administered at successively increasing infusion rates and found only moderate dilator effects. From these observations they concluded that tissue

hyperosmolality could scarcely play an important role in exercise hyperemia. It was emphasized above that in order to reveal the true role of tissue hyperosmolality in exercise hyperemia from intra arterial hypertonic infusion experiments, the infusion rates should be adjusted so as to create the same rapid increase of tissue hyperosmolality as occurs during exercise. In the experiments of Scott *et al.* and Scott and Radawski the hypertonic infusion technique must, however have led to only relatively slow and gradual increases of tissue osmolality therefore, because of the poor resemblance to the osmolar events occurring during exercise itself it is probable that their experiments may permit no valid conclusion about the quantitative role of tissue hyperosmolality in exercise hyperemia. Skinner and Costin (1971) and Gazitúa *et al* (1971) also observed relatively moderate dilator effects in skeletal muscle to hypertonic infusion, but, again, this seems related to the fact that, apparently a fairly slow increase of tissue osmolality was produced in these studies.

As stated in Chapter I, exercise hyperemia is in all probability caused by mutual, synergistic actions of several local metabolic dilator factors. Hyperosmolality appears to be one of these factors, and apparently an important one. Other possible metabolic mediators of functional hyperemia in skeletal muscle have been discussed at great length in different reviews (*e g* Barcroft 1963 Mellander and Johansson 1968 Haddy and Scott 1968 Mellander 1970) and therefore a detailed recapitulation of this subject will not be made. To summarize, it appears that, besides tissue hyperosmolality the following factors deserve special consideration the potassium ion (Kjellmer 1965 *b* and *c*) oxygen lack (*e.g* Fairchild *et al.* 1964) inorganic phosphate (Hilton and Chair 1971) For a detailed discussion of the problem the reader is refered to above-mentioned reviews.

Since the metabolic pathways and, hence, the individual "metabolite" concentrations in skeletal muscle differ with regard to the type of exercise performed there is every reason to believe that the relative importance of the different dilator factors can vary for instance during different phases of the exercise hyperemia response and under different work loads of the skeletal muscle It follows that the conditions under which a proposed mediator of exercise hyperemia plays its main role should be investigated and defined to be able to correctly evaluate its importance for the reaction. From the present study it appears that, although tissue hyperosmolality seems to play a significant role for exercise hyperemia at all levels and in all periods of work, its relative importance seems to be greatest during the development of the vasodilatation and in the subsequent early phases of exercise when the rate of change of tissue osmolality is rapid and furthermore, perhaps particularly in heavy work when the degree of tissue hyperosmolality is most pronounced. The type of detailed investigation performed here may prove useful for evaluating the relative role played by other metabolic mediators. Up to now only tissue hyperosmolality and potassium have been studied in such detail An interesting approach to the problem has been used by Skinner and Costin (1970 1971) who have studied the combined effects of potassium, oxygen

deficiency and hyperosmolality on the resistance vessels in resting skeletal muscle. The results showed that these factors acted synergistically to enhance the hyperemia response.

In Chapter IV the net transcapillary fluid movement into exercising muscle was dealt with and the mechanisms responsible for this fluid transport as well as transcapillary processes involved in the fluid return to the circulatory system after exercise analysed. The investigation was performed on the lower leg, cat muscles which constitute a relatively small part of the total muscle mass (*cf* discussion below). The regional lymph vessels were ligated to be able to study transcapillary events only. The data were discussed at length and in relation to relevant literature and will only be briefly summarized. The fluid flux was found to be related to the work intensity in agreement with Jacobsson and Kjellmer (1964). The rate of fluid movement reached a maximum 2—4 min after commencement of work and was then maintained approximately constant during light and moderate prolonged (15 min) exercise whereas it declined gradually during heavy (4 imp/sec) work. The total amount of fluid that accumulated in the lower leg muscles during 15 min of heavy work averaged no less than about 15 ml/100 g tissue (Fig. 25). The investigation provided concomitant data of fluid transfer into the active muscles of changes of capillary hydrostatic pressure (derived partly from the study reported in Chapter III), of transcapillary hydrodynamic conductivity as determined for the whole muscle region in terms of the transcapillary filtration coefficient (CFC) and of regional osmolar events. The results seemed to make it clear that fluid accumulation in exercising muscle is mainly due to transcapillary fluid osmosis resulting from the work induced tissue hyperosmolality. Thus, at all work intensities, more than 75 per cent of the fluid flux, when maximal, was ascribed to fluid osmosis, the remaining part being due to fluid filtration caused by increased capillary hydrostatic pressure (Figs. 20 and 21). This figure (>75 %) seemed roughly representative also for the cumulative fluid flux during prolonged exercise. The experimental findings indicated that the rate of osmotic fluid movement was directly related to the product of the established veno-arterial osmolar difference and blood flow and this relationship was supported by a theoretical analysis of the data. This analysis suggested that a "weighed mean" of the transcapillary osmotic reflection coefficients for the substances responsible for the interstitial hyperosmolality was approximately constant at different work intensities and that therefore the osmotic fluid flux was directly related to the established transcapillary osmolar gradient and to the overall transcapillary hydrodynamic conductivity in the studied vascular bed. The magnitude of the transcapillary osmolar gradient, in turn, showed a relation to blood flow (capillary flow velocity) through an influence on the transcapillary osmolar extraction. The correlation between work intensity and the rate of total fluid flux could be explained by the mentioned relationships and by a correlation between work intensity, tissue hyperosmolality, blood flow, CFC and increase of capillary hydrostatic pressure, respectively.

A gradual decrease of the transcapillary osmolar gradient was the main factor which limited the fluid movement during prolonged heavy exercise. Rough deductions suggested that a decrease of the transcapillary hydrostatic pressure gradient (increased tissue pressure) and an increase of the colloid osmotic pressure gradient (increased plasma colloid osmotic pressure) also contributed. The decreased transcapillary osmolar gradient was due to a gradually diminishing interstitial hyperosmolality and to an increased arterial osmolality the latter at least partly being due to a "delivery of osmols" from the exercising muscles to the general circulation (cf Chapter II A 1)

The return to the intravascular space after work of the fluid accumulated in the exercising muscles was a slow process which after prolonged heavy exercise required several hours (Fig. 26) Evidence was presented (Fig. 26) to suggest that hydrostatic, colloid osmotic as well as non-protein osmotic forces were responsible for the transcapillary fluid absorption. The non protein osmotic fluid absorption was related to a maintenance for a considerable length of time of the increase of arterial osmolality that occurred during exercise.

It should finally be stressed once more that in the experiments described, lymph drainage was prevented. Therefore, in the intact organism, fluid accumulation in exercising muscle is probably somewhat less pronounced and the rate of fluid return to the circulatory system somewhat greater

Hitherto the discussion has been concerned with *regional* effects of the tissue hyperosmolality in active muscle. A few comments will also be given on some *generalized* effects on cardiovascular hemodynamics that can result from work induced hyperosmolality

First, the effects of hyperosmolality on the plasma volume will be considered. As mentioned, the fluid accumulation in the active muscles averaged about 15 ml/100 g tissue during 15 min of heavy work with a small muscle group (lower leg muscles of the cat) It can be deduced that if fluid accumulated to the same extent in all active muscle during whole body exercise, then the fluid loss from the circulatory system would exceed the entire plasma volume in a relatively short period of time. It is well known, however that in man, plasma volume decreases by at most 15 to 20 per cent, or by some 600 ml, during heavy work with large muscle groups (e g Åstrand *et al.* 1964 Ekelund and Holmgren 1964 Hartley *et al* 1970) This may imply either that fluid accumulation is much more limited when large than small muscle groups are active, and no greater than the actual plasma volume decrease, or if more pronounced, that fluid must be transferred into the circulatory system, for instance from the extravascular space of inactive tissues. A recent study (Lundvall *et al* 1970 1972) in which the fluid accumulation in the active muscle mass and the change of plasma volume were estimated during short term bicycle exercise in man suggested that the latter train of events takes place Thus it was shown that during 6 min heavy exercise on a bicycle ergometer (1200—1500 kpm/min) the volume of the leg in normal males increased so as to indicate, after correction for increased regional

blood volume, an average transcapillary plasma fluid loss into the leg muscles of 4.5 ml/100 g tissue. This fluid accumulation corresponds only to some 50 per cent of that observed in the present study on the lower leg, cat muscles during 6 min of heavy work (*cf* Fig 25) Nevertheless, in the human experiments, the total fluid loss into the entire active muscles mass (~25 kg) was calculated to comprise about 1100 ml. Since the concomitant decrease of plasma volume in man was shown to be about 600 ml, it follows that some 500 ml of fluid must have entered the circulatory system during the work The study indicated that this compensatory fluid gain was accomplished by absorption of extravascular fluid from inactive tissues and partly caused by fluid osmosis resulting from a work induced pronounced *arterial* hyperosmolality Thus, the increase of arterial osmolality averaged no less than 22 mOsm/kg H_2O above the resting control level and seemed mainly caused by delivery of osmols" from the large active muscle mass In the study referred to fluid absorption from inactive tissues was also studied in experimental animals during exercise and in resting humans during arterial hyperosmolality produced by intravenous hypertonic infusion. These investigations suggested that at least half of the fluid gain to the circulatory system in heavy exercise could be ascribed to the increased arterial osmolality and the remainder to a reflex decrease of capillary pressure. It thus appears that the generalized hyperosmolality in the arterial blood resulting from exercise with large muscle groups puts a powerful osmolar mechanism for plasma volume control into play The arterial hyperosmolality helps to keep the plasma volume reduction within tolerable limits partly by causing osmotic fluid absorption from inactive tissues and partly by limiting the fluid loss into the active muscles. The latter effect should result from "osmolar recirculation" which decreases the transcapillary osmolar gradient in the working muscles and may mainly explain why the fluid accumulation was less pronounced in exercising muscle when large muscle groups than when small muscle groups were active. Another factor that can help to explain this difference is the fact that in the animal experiments lymph drainage was prevented. In the experiments in man, on the other hand, return of fluid to the circulatory system *via* the lymph vessels must have reduced to some extent the fluid accumulation.

The work induced hyperosmolality in the active muscle and in arterial blood might also contribute to the general cardiovascular adaptation to exercise. Thus, Lasser *et al* (1960) obtained evidence to suggest that hypertonicity in skeletal muscle affects locally situated "osmoreceptors" and that thereby a reflex in crease of arterial blood pressure and a tachycardia is induced and, in addition, an increased respiratory rate. It was concluded that afferent impulses travel in the peripheral somatic nerves to the central nervous system and induce an in creased discharge rate in sympathetic fibres. The degree of tissue hyperosmolality required to elicit these effects are, however not known. The considerable in-crease of arterial osmolality occurring during exercise with large muscle groups seems also to affect the central circulation. Thus Hauge and Bo (1971) and Bo

et al. (1971) reported that an arterial hyperosmolality of some 25 mOsm/kg H_2O evoked a decrease of pulmonary vascular resistance and concomitantly a constriction of the pulmonary capacitance vessels. Further it has been shown that arterial hyperosmolality of the magnitude just mentioned exerted a positive inotropic action on the heart (Wildenthal *et al.* 1969 *a*) ascribed partly to neurogenic mechanisms and partly to a direct effect on the heart muscle. The latter effect was supported by studies *in vitro* (Wildenthal *et al* 1969 *b* Koch-Weser 1963).

Thus hyperosmolality during exercise may affect the circulation in many different ways. The tissue hyperosmolality in the active muscles promotes the exercise vasodilatation and is mainly responsible for transfer of plasma fluid into the muscle. It might further stimulate local "osmoreceptors" so as to induce a reflex arterial pressure increase and tachycardia. In the general circulation, work induced arterial hyperosmolality can establish an important osmolar control of plasma volume by fluid absorption from inactive tissues and by limiting the transfer of fluid into the active muscle so that plasma volume is maintained within tolerable limits. Further arterial hyperosmolality may promote the action of the heart *via* an inotropic effect, decrease the resistance in the pulmonary circulation and concomitantly constrict the pulmonary veins so as to mobilize blood to the systemic circulation

GENERAL SUMMARY

Experimental evidence suggests that vasodilatation in exercising skeletal muscle is caused by the synergistic actions of several local dilator factors related to the increased tissue metabolism. The present study performed on the cat skeletal muscle, indicates that regional tissue hyperosmolality induced by work is an important mediator of the vasodilatation and of the net transcapillary fluid flux in exercising muscle. This conclusion is based on the following main results

1 Muscle work induced a rapid rise of tissue (venous) osmolality which paralleled the dilator response of the resistance vessels. The extent of hyperosmolality during short term (<5 min) exercise was correlated to the magnitude of the concomitant vasodilator response, both these phenomena, in turn being correlated to the "intensity" of the work. During "heavy" exercise venous osmolality could exceed that at rest by 40—45 mOsm/kg H_2O The hyperosmolality in the extracellular space seemed mainly caused by increased concentrations of sodium and lactate. During more prolonged work the hyperosmolality was maintained except during strenuous work when it tended to decline.

2 Close arterial hypertonic infusions to resting muscle producing graded increases of tissue (venous) hyperosmolality similar to those observed in exercise evoked rapid and graded dilatations of the resistance vessels. Critical analysis indicated that these effects were caused by direct osmotic inhibition of vascular smooth muscle tone by the hypertonicity established in the interstitial space after transcapillary osmolar distribution Under constant pressure perfusion the dilator response was most pronounced in the initial phase of infusion ("peak response") after which there was some partial recovery of vascular tone ("steady state response") possibly caused by a secondary myogenic constrictor effect due to increased transmural pressure. Under constant flow perfusion the osmolar dilator response did not show any such phasic pattern

3 Comparison of the dilator effects of exercise and of hypertonic infusion permitted an approximate quantitative evaluation of the role of tissue hyperosmolality in exercise hyperemia. The relative importance of this factor seemed to be greatest in early phases of work and especially in heavy exercise when the degree of tissue hyperosmolality was most pronounced. The hypertonic infusion effects observed during the "peak response" and during constant flow perfusion could be considered most relevant to the quantitative evaluation. These data suggested that more than 40 per cent of the exercise hyperemia could be ascribed to tissue hyperosmolality during light, and more than 60 per cent during heavy short term exercise. Hyperosmolality may play an important but perhaps somewhat less pronounced role for the dilatation during prolonged work.

4 Studies of the resistance function with regard to changes in resistance distribution within consecutive, delimited parts of the entire vascular tree, and analyses of the precapillary sphincter function and of the capacitance

function showed that the vascular functions were all affected in much the same way during hypertonic infusion to resting muscle as during exercise. Furthermore the highly differentiated patterns of response within various consecutive vascular sections known to be evoked when adrenergic or myogenic stimuli are superimposed on exercise were found to be elicited when these control systems were activated during hypertonic infusion to resting muscle. These findings support the view that tissue hyperosmolality is a causal factor in the exercise vasodilatation.

5 The present results considered together with previous detailed studies of the effects of hyperosmolality on mechanical and electrical activity in isolated spontaneously active vascular smooth muscle suggest that the following train of events occur in exercise: Hyperosmolality develops in the interstitial space of active muscle due to production and release of osmotically active particles from the contracting striated muscle fibres and to osmotic uptake of fluid (water) into these cells; the hyperosmolality in the interstitial space, the immediate environment of the vascular smooth muscle, causes osmotic shrinkage of the smooth muscle cells. The consequent changes of smooth muscle transmembrane ionic concentration gradients and membrane permeabilities to ions lead to inhibition of vascular tone and, hence, to vasodilatation.

6 Concomitant direct or indirect observations in working skeletal muscle of net transcapillary fluid movement, of changes of capillary hydrostatic pressure, of capillary hydrodynamic conductivity and of regional osmolar events indicated that the pronounced fluid accumulation occurring in muscle during exercise is caused mainly (>75 per cent) by osmosis as a result of the work-induced tissue hyperosmolality and that the remainder is due to filtration. The transcapillary fluid absorption into the blood stream *after* exercise seemed caused by hydrostatic, colloid osmotic as well as by non-protein osmotic forces, the latter related to an *arterial* hyperosmolality created during the preceding work period.

7 Some implications of a pronounced arterial hyperosmolality evoked when large muscle groups are active are discussed with regard to effects on general cardio-vascular dynamics.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Medical Research Council (B72 14X 2210-06A) and from the Faculty of Medicine, University of Lund, Sweden.

I have had the privilege to work under the stimulating guidance of Professor Stefan Mellander who has been my teacher and who introduced me to this field of research I wish to express my sincere gratitude to him for his invaluable active help

My thanks are due to Civ Ing Lars-Åke Ledebo and to Professor Börje Johansson for valuable criticism and help.

I am much indebted to Mrs. Tora Zadig Johansson Miss Margareta Rahm, Miss Susanne Carlin, Miss Ann-Christin Isaksson, Miss Christine Boström and Mr Olle Hammar for skilled technical assistance, typing of the manuscripts and preparation of the figures.

REFERENCES

ABLAD, B. and S. MELLANDER, Comparati e effects of hydralazine, sodium nitrite and acetylcholine on resistance and capacitance blood vessels and capillary filtration in skeletal muscle in the cat. *Acta physiol. scand.* 1963 58 319—329

ALEXANDER, R. S., The peripheral venous system. In: *Handbook f Physiology* ed. by W F Hamilton and P Dow The Williams & Wilkins Co., Baltimore, 1963 Sect. 2, vol 2, pp. 1075—1098.

ARTURSON, G and I. KJELLMER, Capillary permeability in skeletal muscle during rest and activity *Acta physiol. scand.* 1964 62. 41—45.

ARTURSON G and S. MELLANDER, Acute changes in capillary filtration and diffusion in experimental burn injury *Acta physiol. scand.* 1964 62 457—463

ARVILL, A., B. JOHANSSON and O. JONSSON Effects of hyperosmolarity on the volume of vascular smooth muscle cells and the relation between cell volume and muscle activity *Acta physiol. scand.* 1969 75 484—495

ÅSTRAND P-O T E CUDDY B SALTIN and J STENBERG, Cardiac output during submaximal and maximal work. *J ppl. Physiol.* 1964 19 268—274

BARCROFT H., Circulation in skeletal muscle. In: *Handbook f Physiology* ed. by W F Hamilton and P Dow The Williams & Wilkins Co., Baltimore, 1963 Sect. 2, vol. 2, pp. 1353—1385

BARCROFT J and T KATO Effects of functional activity in striated muscle and the submaxillary gland. *Phil. Trans. roy Soc. Ser B Biol. Sci.* (Lond.) 1915 207 149—182.

BERGSTRÖM, J G GUARNIERI and E. HULTMAN Carbohydrate metabolism and electrolyte changes in human muscle tissue during heavy work. *J ppl. Physiol.* 1971. 30 122—125

BEVEGÅRD B. S. and J T SHEPHERD Regulation of the circulation during exercise in man. *Physiol. Rev* 1967 47 178—213

BØ, G A. HAUGE and G. NICOLAYSEN, Hyperosmolarity and pulmonary vascular capacitance. *Acta physiol. scand.* 1971. 82 375—381.

BURTON A. C., *Physiology and biophysics f the circulation.* Year book medical publishers. Chicago. 1966.

COBBOLD A., B. FOLKOW I. KJELLMER and S. MELLANDER, Nervous and local chemical control of pre-capillary sphincters in skeletal muscle as measured by changes in filtration coefficient. *Acta physiol. scand.* 1963 57 180—192.

DAUGHERTY R. M. Jr J B. SCOTT J M. DABNEY and F J HADDY Local effects of O_2 and CO_2 on limb, renal, and coronary vascular resistance. *Amer J Physiol.* 1967 *213* 1102—1110.

DIANA, J N Capillary pressure and filtration coefficient during isoproterenol infusion. *Amer J Physiol.* 1970. *219* 1574—1584

DORNHORST A. C. and R. F WHELAN The blood flow in muscle following exercise and circulatory arrest: the influence of reduction in effective local blood pressure, of arterial hypoxia and of adrenaline. *Clin. Sci.* (Lond.) 1953 *12* 33—40.

DULING B. R. and R. M. BERNE, Longitudinal gradients in periarteriolar oxygen tension. A possible mechanism for the participation of oxygen in local regulation of blood flow *Circulat Res.* 1970 *27* 669—678.

EKELUND L.-G and A. HOLMGREN Circulatory and respiratory adaptation during long term, non-steady state exercise, in the sitting position. *Acta physiol. scand.* 1964 *62.* 240—255

FAIRCHILD H. M., J ROSS and A. C. GUYTON, Failure of recovery from reactive hyperemia in the absence of oxygen. *Amer J Physiol.* 1966. *210* 490—492.

FOLKOW B. and H. D HALICKA, A comparison between "red" and "white" muscle with respect to blood supply capillary surface area and oxygen uptake during rest and exercise. *Microvascul. Res.* 1968 *1* 1—14

FOLKOW B., O. LUNDGREN and I. WALLENTIN Studies on the relationship between flow resistance, capillary filtration coefficient and regional blood volume in the intestine of the cat. *Acta physiol. scand.* 1963 *57* 270—283

FOLKOW B. and S. MELLANDER, Measurements of capillary filtration coefficient and its use in studies of the control of capillary exchange. In: *Capillary Permeability Alfred Benzon Symposium II* ed. by C. Crone and N A. Lassen. Munksgaard, Copenhagen. 1970. pp. 614—623

FOLKOW B. and E. NEIL, *Circulation.* Oxford University Press, London and Toronto, 1971.

FOLKOW B., R. R. SONNENSCHEIN and D L. WRIGHT Loci of neurogenic and metabolic effects on precapillary vessels f skeletal muscle. *Acta physiol. scand.* 1971. *81* 459—471

FROHLICH, E. D Prolonged local and systemic hemodynamic effects of hyperosmotic solutions. *Archs int. Pharmacodyn* 1966. *161* 154—166.

GAZITÚA, S J B. SCOTT B. SWINDALL and F J HADDY Resistance responses to local changes in plasma osmolality in three vascular beds. *Amer J Physiol.* 1971 *220* 384—391

GOLDBERG N D J V PASSONNEAU and O H. LOWRY Effects of changes in brain metabolism on the levels f citric acid cycle intermediates. *J biol. Chem.* 1966. *241* 3 3997—4003

GRAY S. D E. CARLSSON and N C. STAUB, Site f increased vascular resistance during isometric muscle contraction. *Amer J Physiol.* 1967 *213* 683—689

GRAY S D J LUNDVALL and S. MELLANDER, Regional hyperosmolality in relation to exercise hyperemia. *Acta physiol. scand* 1968 *73* 11A—12A.

GRAY S D J LUNDVALL and S. MELLANDER, Unpublished observations.

GUYTON A. C., Interstitial fluid pressure II. Pressure-volume curves of interstitial space. *Circulat. Res* 1965 *16* 452—460.

GUYTON, A. C., *T xtbook f medical physilogy* W B. Saunders Company Philadelphia and London, 1966.

HADDY F J A. G. RICHARDS, J L. ALDEN and M. B VISSCHER, Small vein and artery pressures in normal and edematous extremities of dogs under local and general anesthesia. *Amer J Physiol* 1954 *176* 355—360.

HADDY F J and J B. SCOTT Metabolically linked vasoacti chemicals in local regulation of blood flow *Physiol. Rev* 1968, *48* 688—707

HARTLEY L. H., B. PERNOW J HÄGGENDAL, J. LACOUR, J DE LATTRE and B SALTIN, Central circulation during submaximal work preceded by heavy exercise. *J appl Physiol.* 1970, *29* 818—823.

HAUGE, A. and G. BØ Blood hyperosmolality and pulmonary vascular resistance in the cat. *Circulat. Res.* 1971 *XXVIII* Suppl. I 371—376.

HILTON, S M. and B. CHIR, A new candidate for mediator of functional vasodilatation in skeletal muscle. *Circulat. Res* 1971 *XXVIII* Suppl. I 70—72.

HIRVONEN L. and R. R. SONNENSCHEIN Relation between blood flow and contraction force in active skeletal muscle. *Circulat. Res.* 1962, *10* 94—104

HYMAN, C. and W H. WONG Nature and mechanism of the restoration of normal blood flow after exercise and ischemia. In: *Circulation in skeletal muscle* ed. by O Hudlická Oxford Pergamon. 1968. pp. 205—213

JACOBSSON, S. and I. KJELLMER, Accumulation of fluid in exercising skeletal muscle *Acta physiol. scand* 1964 *60* 286—292.

JOHANSSON, B., Permeability characteristics of vascular smooth muscle cells as revealed by their osmotic responses to non-electrolytes. *Acta physiol. scand* 1969 *77* 282—297

JOHANSSON B. and B. LJUNG Spread of excitation in the smooth muscle of the rat portal vein. *Acta physiol. scand* 1967 *70* 312—322.

JOHANSSON, B. and O JONSSON Cell volume as a factor influencing electrical and mechanical activity of ascular smooth muscle. *Acta physiol. scand.* 1968 *72* 456—468

JONSSON O Extracellular osmolality and ascular smooth muscle activity *Acta physiol. scand* 1970, Suppl. 359 1—48

KARNOVSKY M. J The ultrastructural basis of capillary permeability studied with peroxidase as a tracer *J cell. Biol.* 1967 *35* 213—236.

KARNOVSKY M. J Morphology of capillaries with special reference to muscle capillaries. In *Capillary Permeability Alfred Benzon Symposium II* ed. by C. Crone and N A. Lassen. Munksgaard, Copenhagen. 1970. pp. 341—350.

KJELLMER, L., The effect of exercise on the vascular bed of skeletal muscle. *Acta physiol. scand* 1964 *a*, *62* 18—30.

KJELLMER, I., An indirect method for estimating tissue pressure with special reference to tissue pressure in muscle during exercise. *Acta physiol scand* 1964 *b* *62* 31—40.

KJELLMER, I On the competition between metabolic vasodilatation and neurogenic vasoconstriction in skeletal muscle. *Acta physiol. scand* 1965 *a*. *63* 450—459

KJELLMER, I., The potassium ion as asodilator during muscular exercise. *Acta physiol. scand.* 1965 *b* *63* 460—468

KJELLMER, I., Studies on exercise hyperemia. *Acta physiol. scand* 1965 *64* Suppl. 244 1—27

KJELLMER, I. and H. ODELRAM The effect of some physiological vasodilators on the vascular bed of skeletal muscle. *Acta physiol. scand* 1965 *63*. 94—102.

KOCH WESER, J Influence of osmolarity of perfusate on contractility of mammalian myocardium. *Amer J Physiol.* 1963 *204* 957—962.

KONTOS, H. A., D W RICHARDSON and J L. PATTERSON J Vasodilator effect of hypercapnic acidosis on huma forearm blood vessels. *Amer J Physiol.* 1968 *215* 1403—1405

KRAMER, K., F OBAL and W QUENSEL, Untersuchungen über den Muskelstoffwechsel des Warmeblüters. III Mitteilung. Die Sauerstoffaufnahme des Muskels während rhytmischer Tätigkeit. *Pflügers Arch ges. Physiol.* 1939 *241* 717—

LANDIS, E. M., Capillary pressure and capillary permeability Physiol. Rev 1934 14 404—481.

LANDIS, E. M. and J R. PAPPENHEIMER, Exchange of substances through the capillary walls. In: *Handbook of Physiology* ed. by W F Hamilton nd P Dow The Williams & Wilkins Co., Baltimore, 1963 Sect. 2, vol. 2, pp 961—1034

LANDIS, E. M. and L. E. SAGE, Fluid movement rates through walls of single capillaries exposed to hypertonic sol tions. *Amer J Physiol.* 1971, *221* 520—534

LASSER, R. P M. R. SCHOENFELD D F ALLEN and C. K. FRIEDBERG Reflex circulatory effects elicited by hypertonic and hypotonic solutions injected into femoral and brachial arteries of dogs *Circulat. Res.* 1960. *VIII* 913—919

LOWRY O H., J V PASSONNEAU F X. HASSELBERGER a d D W SCHULZ, Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain. *J Biol. Chem.* 1964 *239* 18—30.

LUNDGREN O and S. MELLANDER, Augmentation of tissue-blood transfer of solutes by transcapillary filtration and absorption. *Acta physiol. scand.* 1967 *70* 26—41

LUNDVALL, J Further studies on regional hyperosmolality in relation to exercise hyperemia. *Acta physiol scand* 1969 Suppl. 330. 98.

LUNDVALL, J S. MELLANDER and H. SPARKS, Myogenic response of resistance vessels and precapillary sphincters in skeletal muscl during exercise. *Acta physiol scand* 1967 *70* 257—268

LUNDVALL, J S. MELLANDER, H. WESTLING and T WHITE, Dynamics of fluid transfer between the intra and extravascular compartments during exercise. *Acta physiol. scand* 1970 *80* 31A—32A.

LUNDVALL, J S MELLANDER, H. WESTLING and T WHITE, Fluid transfer between blood and tissues during exercise. *Acta physiol scand.* 1972. *00* 000—000.

LUNDVALL, J S. MELLANDER and T WHITE, Hyperosmolality and vasodilatation in huma skeletal muscle. *A ta physiol. scand.* 1969 *77* 224—233

MAJNO G Ultrastructure f the vascula membrane. In *Handbook f Physiology* ed. by W F Hamilton and P Dow The Williams & Wilkins Co., Baltimore, 1965 Sect. 2, vol. 3 pp. 2293—2375

MARSHALL, R. J and J T SHEPHERD Effect f injections f hypertonic solutions on blood flow through the femoral artery of the dog. *Amer J Physiol.* 1959 *197* 951—954

MEISELMAN H. J E. W MERRILL, E. R. GILLILAND G A. PELLETIER and E. W SALZMAN Influence of plasma osmolarity on the rheology f human blood. *J ppl. Physiol* 1967 *22*. 772—781

MELLANDER, S., Comparati e studies on the adrenergic neuro-hormonal control of resi tance and capacitance blood vessels in the cat. *Acta physiol. scand.* 1960 *50* Suppl. 176, 1—86.

MELLANDER, S., Comparative effects of acetylcholine, butyl-nor-synephrine (Vasculat) noradrenaline, an ethyl-adrianol (Effontil) on resistance, capacitance and precapillary sph ncter vessels and capillary filtration in cat skeletal muscle. *Angiologica.* 1964. *3* 77—99

MELLANDER, S., Systemic circulation. Local control. *A. Rev. Physiol* 1970. *32* 313—344

MELLANDER, S., Interaction of local and nervous factors in ascular control. *Angiologica* 1971 *8*. 187—201.

MELLANDER, S. and B. JOHANSSON Control of resistance, exchange and capac tance functions in the peripheral circulation. *Pharmacol Rev* 1968 *20* 117—196

MELLANDER, S., B. JOHANSSON S. GRAY O. JO SSO J. LU D ALL and B. LJUNG, The effects of hyperosmolarity on intact and isolated vascular smooth muscle Possible role in exercise hyperemia. *Angiologica.* 1967 *4* 310—322.

MELLANDER, S. and J. LUNDVALL, Role of tissue hyperosmola ty in exe se hyperem a *Circulat. Res.* 1971. *XXVIII and XXIX* Suppl. I 39—45

MILLER, R. G., *Simultaneous statistical inference* New York, McGraw-Hill Co 1966

MYERS, H. A. and C. R. HONIG, Influence of initial resistance on mag [illegible] to vaso-motor stimuli. *Amer J Physiol.* 1969 *216*. 1429—1436.

OBERG, B., The relationship between acti constriction and passive recoil of the [illegible] at various distending pressures. *Acta physiol. scand* 1967 *71* 233—247

OVERBECK, H. W and G. J GREGA, Response of the limb [illegible] to intrabrachial arterial infusions of hypertonic dextrose or hypertonic [illegible] *Circulat. Res.* 1970. *XXVI* 717—731

OVERBECK, H. W J I MOLNAR and F J HADDY Resistance to [illegible] through the vascular bed of the dog forelimb. *Amer J Cardiol.* 1961 *8* 533—541

PAPPENHEIMER, J R., Osmotic reflection coefficients in capilla y membranes In *Cap* [illegible] *Permeability Alfred Benzon Symposium II* ed. by C. Crone and N. A. Lassen [illegible] Copenhagen. 1970. pp. 278—286.

PERL, W Modified filtration-permeability model of transcapillary transport [illegible] the Pappenheimer poe paradox? *Microvascul Res.* 1971 *3* 233—251.

RAND P W and E. LACOMBE, Hemodilution, tonicity and blood [illegible] 1964 *43* 2214—2226.

REIN, H., Die interferenz der asomotorischen Regulationen. *Klin Wschr* [illegible] 1415

REMENSNYDER, J P J H. MITCHELL and S. J SARNOFF [illegible] during muscular activity Observations on influence of carotid [illegible] *Circulat. Res.* 1962. *11* 370—380.

SCHMID-SCHÖNBEIN H. and R. WELLS, Rheological [illegible] crenation. *Pflügers Arch ges. Physiol.* 1969 *307* 59—69

SCOTT J B. and D RADAWSKI, Role f hyperosmolarity in the [illegible] reacti hyperemia. *Circulat Res.* 1971 *XXVIII* Suppl. I 26—32

SCOTT J B M. RUDKO D RADAWSKI and F J HADDY [illegible] Mg^{++} and O_2 in local blood flow regulation. *Amer J Physiol.* [illegible] 311—[illegible]

SIGGAARD-ANDERSEN O Blood acid-base alignment nomogram. *Scand J clin. Lab Invest* 1963 *15* 211—217

SKINNER, N S. J and J C. COSTIN Role of O_2 and K^+ in abolition of sympathetic vasoconstriction in dog skeletal muscle. *Amer J Physiol.* 1969 *217* 438—444

SKINNER, N S. Jr and J C. COSTIN Interactions of vasoactive substances in exercise hyperemia O_2, K^+ and osmolality *Amer J Physiol.* 1970. *219* 1386—1392.

SKINNER, N S. Jr and J C. COSTIN Interactions between oxygen, potassium, and osmolality in regulation of skeletal muscle blood flow *Circulat Res.* 1971 *XXVIII* Suppl. 1 73—85

SMAJE, L., B W ZWEIFACH and M. INTAGLIETTA, Micropressures and capillary filtration coefficients in single vessels of the cremaster muscle of the rat. *Microvascul. Res.* 1970. 2. 96—110.

SOMLYO A. P and A. V SOMLYO Vascular smooth muscle. 1 Normal structure, pathology biochemistry and biophysics. *Pharmacol. Rev* 1968 *20* 197—273

STAINSBY W N and M. J FREGLY Effect of plasma osmolality on resistance to blood flow through skeletal muscle. In *Circulation in skeletal muscle* ed. by O Hudlická, Oxford: Pergamon. 1968 pp 315—322.

SÖDERSTRÖM, N Hemolysis by hypertonic solutions of neutral salts. *Acta physiol. scand.* 1944 *7* 56—68.

UCHIDA, E., D F BOHR and S. W HOOBLER, A method for studying isolated resistance vessels from rabbit mesentery and brain and their responses to drugs. *Circulat. Res.* 1967 *21* 525—536.

VARGAS, F and J A. JOHNSON An estimate of reflection coefficients for rabbit heart capillaries. *J gen. Physiol.* 1964 *47* 667—677

WIEDEMAN M. P P ttern of the arteriovenous pathways. In *Handbook of physiology* ed. by W F Hamilton and P Dow The Williams & Wilkins Co., Baltimore, 1963 Sect. 2, vol. 2, pp. 891—933

WILDENTHAL, K., D S. MIERZWIAK and J H. MITCHELL, Acute effects of increased serum osmolality on left ventricular performance. *Amer J Physiol.* 1969 *a*. *216*. 898—904

WILDENTHAL, K., C. L. SKELTON and H. N COLEMAN III, Cardiac muscle mechanics in hyperosmotic solutions. *Amer J Physiol.* 1969 *b* *217* 302—306.

WRIGHT D L. and R. R. SONNENSCHEIN Relations among activity blood flow and vascular state in sk letal muscle. *Amer J Physiol.* 1965 *208* 782—789

WRIGHT D L and R. R. SONNENSCHEIN Muscle force and electromyogram with alteration in flow and composition f blood. *Amer J Physiol* 1969 *216* 1075—1080.

YUDILEVICH, D L. and O A. ALVAREZ, Water sodium, and thiourea transcapillary diffusion in the dog heart. *Amer J Physiol* 1967 *213* 308—314

ZWEIFACH, B. W and M. INTAGLIETTA, Mechanics of fluid movement across single capillaries in the rabbit. *Microvascul. Res.* 1968 *1* 83—101

ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 380

Seasonal variations in the physiology and biochemistry of the european hedgehog (Erinaceus europaeus) including comparisons with non hibernators guinea pig and man

Edited by

BENGT W JOHANSSON

and

JEROME B SENTURIA

MALMÖ 1972

Fig 0.1

A magnificent picture of the Copernican system from Andreas Cellarius atlas Harmonia Macrocosmica, sev Atlas Universalis et novus totis Universi creati Cosmographiam Generalem et Novam exhibens, Joannem Janssonium Amsterdam 1661

Andreas Cellarius was a German mathematician geographer and cosmographer in Horn Holland

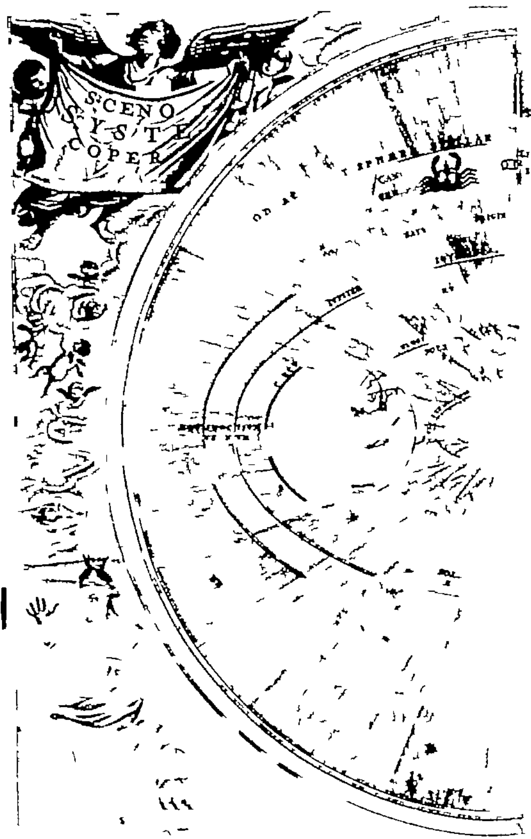
The Copernican hypothesis was published in 1543 in De revolutionibus orbium coelestium Libri VI by Nicolaus Copernicus and it was not until 1687 when Newton published Philosophiae naturalis principia mathematica that the Copernican system was generally accepted

Between 1616-1757 Copernicus work was forbidden by the church.

The Copernican system explains the seasons in a very simple manner According to this system seasonal variations are due to the $66^{1}/_{2}°$ relationship between the rotational axis of the earth and the plane of the earth s orbit around the sun

In this picture the earth is placed in the four seasonal positions Aeqvinoctivm Vernvm (vernal equinox) Solstitivm Aestivvm (summer solstice) Aeqvinoctivm Avtvmnale (autumn equinox) and Solstitivm Brvmale (winter solstice)

The other planets known in 1661 Mercury Venus Mars Jupiter and Saturn complete the picture of our solar system The distal circle represents the Zodiac The signs of the Zodiac through which the sun passes are Aries (ram) in the vernal equinox, Cancer (crab) in the summer solstice Libra (scales) in the autumn equinox, and Capricornus (unicorn) in the winter solstice


SCENO
SYSTE
COPER



GRAPHIA
MATIS
NICANI

LIST OF CONTRIBUTORS

Björn Åkesson, M.D
Division of Physiological Chemistry
Chemical Center
University of Lund
P O Box 740
S-220 07 Lund
Sweden

Anders Bjernstad, M.D
Coagulation Laboratory
General Hospital
S-214 01 Malmö
Sweden

Miss Barbro Eklund
Heart Laboratory
Department of Medicine
General Hospital
S-214 01 Malmö
Sweden

Hans Ericsson, M.D
Coagulation Laboratory
General Hospital
S-214 01 Malmö
Sweden

Bengt G Johansson M.D
Department of Clinical Chemistry
General Hospital
S-214 01 Malmö
Sweden

Bengt W Johansson, M.D
Heart Laboratory
Department of Medicine
General Hospital
S-214 01 Malmö
Sweden

Kai C. Nielsen, M.D
Department of Neurosurgery A
University Hospital
S-221 85 Lund
Sweden

*Sven-Olle R. Olsson, Ph.D.
Zoophysiological Institute
University of Lund
Lund Sweden

Christer Owman, M.D
Institute of Anatomy and Histology
University of Lund
Lund, Sweden

Maurizio Pandolfi, M.D
Coagulation Laboratory
General Hospital
S-214 01 Malmö
Sweden

Jerome B Senturia, Ph.D
Department of Biology and Health Sciences
The Cleveland State University
Euclid Avenue at 24th Street
Cleveland Ohio 44115
U.S.A.

**Wilfried von Studnitz, M.D
Department of Clinical Chemistry
General Hospital
S-214 01 Malmö
Sweden

Jan Thorell, M.D
Isotope Laboratory
General Hospital
S-214 01 Malmö
Sweden

*Present Address
AB Ferrosan
Fack, S-201 10 Malmö 1
Sweden

**Present Address:
Nussbaumstrasse 14
D-8 München 2
Germany

Tabl of contents

PREFACE

Despite steadily increasing interest i the subject hibernation is still challenge to scientists The reasons for thi ar manif ld One reason is that natural mammalian hibernation occurs in species belonging to diverse taxonomi groups; therefore hibernation i golden hamsters is not exactly the same as hibernation i bats This i eflected i the different proneness to enter the hibernating state shown by these animals Results obtained in one species can thus not be transferred directly to other species Another reason is that ther is not one ingle factor which haracteri es the hibernator when compared to other mammals. Not only exogenous factors such as ambient temperature ar of importanc for hibernation but endogenous factors such as an increase of fat depots are important as preparation for hibernation Equally important are inborn qualities such as the ability of the cardiovascular and nervous system to function at temperatures just above 0° C Against this background we started an extensive study i one hibernating species the hedgehog collected in one local area i southern Sweden omparing relevant parameters obtained from varying phases of the hibernating cycle Samples from guinea-pig and man were tudied simultaneously thus enabling us to compare dat obtained i hibernating and non-hibernating species The cooperation with investigators from different disciplines provided us with great amount of information This interdisciplinary approach brings together highly qualified staff trained in differ- ent areas of the biologi al sciences and provides integrated and compr hensive studies whi h i of great value especially i hibernation research because the whol fi ld of physi logy and biochemistry is of relevanc i the study of hibernation I hope the results reported i this supplement will give birth to new ideas and impulses and stimulate to furthe research with the purpose to solve the hibernation problem.

Bengt W Johansson

Chapter 1

G n r l Int od tio

by

Bengt W Johansson and Jerome B Senturia

I General Background to Hibernation

Hibernation has been defined in many ways (Hoffman 1964) Natural mammalian hibernation can be concisely defined as depressed metaboli stat generally associated with the winter season I this state the mammalian hibernator no longer regulates body temperature in the 35°-39 C "normal range but permits its body temperature to fall to few degrees above ambient temperature It does not permit its body temperature to fall to 0° C The hibernator has the ability to rewarm spontaneously to the "normal body temperature The animal does this by endogenous heat production and does not equire added heat from the environment for this aroused process Estivation is apparently imilar if not identical process to hibernation with the exception that estivation i process whi h is associated with the summer season

The term hibernation has been applied to all manner of dormancy conditions included i this term has been wi ter dormancy of insects amphibians eptiles and other animal These states are often confused with natural mammalian hibernation The key difference i that these animals rely on the environment for temperature regulation whil mammalian hibernators are endotherml that is they are capabl of maintai ing constant high body temperature through metabolic heat production

The hibernator derives physiological advantage from this process. He i abl to survive during periods when environmental conditions sever ly educ the availability of food Thus it is probabl that the reduced energy requirement of hibernation and estivation is the factor which has given this trait evolutionary survival value (Levins 1969)

Hibernation i seasonal ycl In nature it is coupled to the limati hanges associated with evolution of th earth around the sun (see fig 0 1) W can di ide the hibernation cycl into five physiological periods First the summer or active stat i which the hi bernator's physiological esponses ar similar to those of non-hibernator Second period of preparation for hibernation i which the animal increases i food intake and weight

and number of endocrine hanges occur Third entry I to hibernation which involves hanges from the active state into the depressed metabolic state Fourth hibernation or maintenance of the organism during the period of lowered body temperatur and associated low metabolic rates Finally periodic or terminal arousal from hibernation triggered either by stimuli from the environment or from endogenous clues (Pengelley 1967) Arousal is function of internal generation of heat especially by the brown fat and heart whil shi ering plays a rol late in arousal Arousal is marked by differential distribution of blood such that the heart-brai axi is warmed early in arousal

Tissues from hibernators function at lower temperatures than those from non-hibernators (Willis 1967) Nerves from hamsters conduct at 3 4 C (Chatfield et l 1948) The isolated hearts of hibernators beat to temperatures near 0 C (Lyman and Blinks 1959 Senturia t l 1970); corresponding temperatures in non-hibernating animals are higher Hearts of hibernating animal seem less sensitive to ventricular fibrillation than non-hibernators (Johansson 1967)

During the process of hibernation two sets f changes or apparent First hanges due to the lowering of body temperatures and the esultant metabolic depression and second hanges which represent specifi adaptations to the conditions of hibernation It has bee shown that the intrinsi rate-temperature response of the isolated heart of bats and ground squirrel (Mi hael and Menaker 1963 Senturi t l 1970) I constant throughout the year Indi ating that reduction I heart rat during hibernation may be passive temperature related phenomenon Lyman and his co-workers (Lyma 1965) have shown that heart rat I not a passive function of temperature I the whol animal during entry and arousal This points t modifi cation I the extrinsi ontrol on the heart during the entry and arousal processes

Considerabl differences exist between species of hibernators There are hibernators I such diverse taxonomi groups as I tI o includi g hedgehogs and tenrecs d t including many species of ground squirr I marmots hamsters doormi and the birch mouse Sicista I o e limited to some species of bear and chiropterans including the following families of bats Rhinolophidae and Vespertilionidae (Davies 1970 Lyman 1970) These differences between species are compounded by the fact that the sam species I the hands of different investigators have led t confli ting results

II Background to the Specifics of the Study

Pengelley (1967) has hown annual rhythms in body weight I ground squirrels Seasonal changes I weight have been noted I many species of hibernators (Pengelley 1967) Fatteni g in preparation for winter has also been shown I animal whi h do not hibernat (Heroux 1961) Seasonal changes I weight of organs have been shown previously I hibernators (Solvi 1967 Kayser 1961) I non-hibernators seasonal changes I the weight of reproductive organs has

been noted I both hibernating and non-hibernating mammal the occurence of seasonal sexual cycles I well documented (Asdell 1946) The photoperiodic natur of control of many of these cycles has been elucidated (Farner 1961)

Earlier studies on the lectrocardiograms of hibernators are summari ed by Johansson (1967) One of the most marked features of hibernation is the extreme bradycardi Hong *et al* (1967) showed seasonal changes (presumably related to water temperature) I ECGs from diving Korean ama her too bradycardia I lary featur Davies (1967) showed increased blood pressure I winter I man living I the Antarcti

Seasonal variations I blood ions have been studied from many aspects including hibernation (Fisher and Manery 1967) and nutrient availability (Barakat and Hassanei 1969) The results of these studies are conflicting and discussed I more detail I Chapter 5 on blood ions.

Recent studies on blood gases I hibernators have been performed I the hedgehog by Clausen (1966) Clausen and Ersland (1968) and Bartels *et al* (1969) and I marmots (*Marmota flaviventris* and *M. monax*) by Goodrich (1972) The data from various investigators on acid base balanc I hibernators I summarized by Goodri h (1972) Blood pH values I hibernators at 5° C vary from 7 27 I ground squirrels to 7 38 I European hedgehogs to 7 44 in hamsters Blood P_{CO_2} values vary from 32 to 55 mm Hg in the same animals Interest I blood gas levels I hibernation has been generated by the data on changes I metabolic rat and by observations on the tolerance of hibernators to hypoxia (Burlington *et* I 1971 Biörck and Johansson 1957)

During hibernation there is no intake of food Thi has prompted much interest I energy sources during hibernation and arousal (South and House 1967) Glycogen may increase prot ins decrease (Willi 1967) and fat decreases (South and House 1967) It has been noted (Fisher and Manery 1967) that hibernators have higher blood urea (and NPN) levels and tolerate them better than non-hibernators I bears (Nelson *et al* 1971) blood urea does not increase during hibernation Nelson *et* I (1971) lso note that the blood level of urea was generally less than 33 mg/100 ml of plasma Clearly the hibernator must possess adaptations to deal with the nitrogenous wast products of metabolism.

Carbohydrat metabolism of hibernators has been studied from several aspects Musacchi and Bralnat (1967) using *in vivo* techniques onfirmed that hamsters and ground squirrels have reduced transport of intestinal glucose during hibernation and that levels of glucose transport increases to non-hibernating levels soon after arousal Tashima *et al* (1970) using radioglucose studied the fate of glucose I hibernating ground squirrel Their results indicated what had been suggested earlier (South and House 1967) that I hibernation fatty acid synthesis and CO_2 production were decreased lative to incorporation I to glyceride prot I and glycogen Thi confirms other observations egarding predominance of glycolysis during hibernation and the increased synthesis of glycogen from lactic acid Whitten and

Klain (1968a) give evidence for gluconeogenesis from protei and decreased protein synthesis during hibernation and arousal Among the metabolic control mechanisms whi h might be operative are coupling of oxidative phosphorylation Isoenzyme fluctuations and the operation of metabolic hormones Kayser (1964) suggested that the coupling of oxidative phosphorylation is higher in mitochondria from hibernators Simmonds et al (1971) examined the LDH isoenzymes i ground squirrels and numerous studies have been undertaken to study the rol of the endocrine glands i hibernators (Hoffman 1964)

The fact that most hibernators fatten in the fall indicates changes i lipid metabolism *Galster and Morrison (1966) noted that serum values for total lipid α-lipid β-lipid and* hylomicrons wer lowest in spring and highest in the fall Baumber and Denyes (1963) showed that hibernating hamsters incorporated acetat C^{14} i to lipid at greater rat than non-cold acclimated hamsters

One control mechanism which has been implicated as being crucial to hibernators has been the autonom nervous system. Lyman and O'Brien (1963) concluded that whil th parasympatheti nervous system acts i a regulatory capacity during entrance into hibernation it i not essenti l to the completion of the hibernation cycle The parasympatheti nervous system on the other hand is required for maintenanc of blood flow during hibernation and control duri g hibernation A major proportion of the heat necessary for arousal from hibernation is produced by the brown fat (Hayward and Lyman 1967 Smith and Horwitz 1969) The production of this heat i apparently triggered by norepinephrine from sympatheti nerve endings (Hayward 1968) Ni lsen and Owman (1968) have shown that hibernators lack adrenergi innervation to the myofibril i the myocardium i ontrast to non-hibernators. Thi observation has been disputed by Angelakos et l (1971)

It is often observed that the blood of hibernators lots lower than that from non-hibernators Since blood flow i reduced during hibernation mechanisms to delay lot formation Bärck et al (1962) or increase fibrinolysi are important

In the present study we have attempted to ompare everal variables as function of season in hibernators and non-hibernators The use of common material gives us the ability to compare the results of different investigators directly The interdisciplinary approach represented here gives us broad expertise and speci li ed methods to examine the problems associated with seasonal adaptation to the environment Above ll the interdisciplinary approach has allowed an exchange of ideas not easily obtained withi one laboratory

Chapter 2

G ne l M th ds nd M teri ls

by

Jerome B Senturia Barbro Eklund and Bengt W Johansson

The following methods and materials wer common to all the studies reported i this volume

Biological materials Three species were used i this study The european hedgehog (Erinaceus europaeus) was selected as an availabl hibernator of appropriat siz which ould be kept well i the laboratory The guinea-pig (Cavi porcellus) was used as representative non-hibernator Man (Homo sapiens) was used to compar some of the blood hemistry findings seen i hedgehogs and guinea-pigs Thus three different orders of mammal are represented i thi study: Insectivora Rodentia and Primata respectively

Feedi g and caging: The hedgehogs were kept i semisheltered enclosures i large groups (25-60) and provided with hay as bedding No attempt was made to control temperature in these enclosures and thei ambient temperature was that of the natural environment

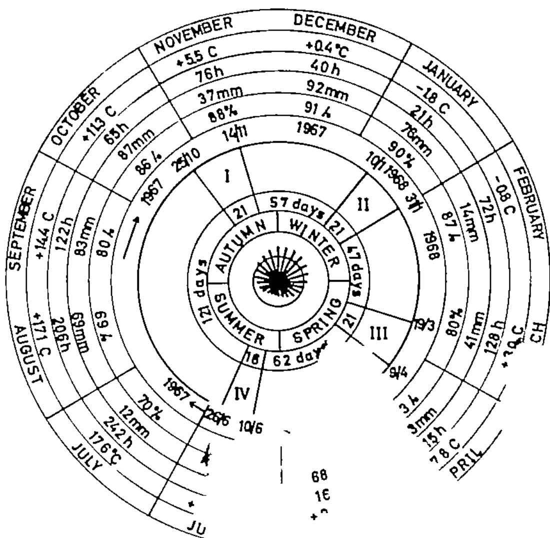
The guinea-pigs were kept i ordinary metal laboratory cages (30 cm 40 cm 30 m) indoors i groups of 2-3 or individually The room was heated i winter but not ooled i summer Both species received natural light cycl

The seasonal changes in temperatur and other meteorological factors are shown in figure 2 1

The hedgehogs were fed tabl scraps from the central kitchen of the Malmö General Hospital They were not fed during the winter (hibernating period) The guinea-pigs were fed laboratory animal pellets supplemented with gras lippings and fresh vegetables

No attempt was made to control the diet or housing onditions of the human subjects The human subjects consisted of four mal physicians two mal zoologists one male laboratory assistant two femal medical secretaries and four femal laboratory assistants.

Time of sampling: T compare the annual seasonal phases of the hibernation cycle samples wer taken in four seasonal periods Each day of sampling period no mor than two samples of each species were taken i order to mi imize the effect of circadian hanges the samples were taken 5 to 6 hours after sunrise for the animals and at 8-9 m. for man



NOVEMBER
DECEMBER
JANUARY
FEBRUARY
OCTOBER
SEPTEMBER
AUGUST
JULY
+5.5 C
76h
37mm
88%
14/11
+0.4°C
40h
92mm
91%
1967
-1.8 C
21h
78mm
90%
10/1 1968
-0.8 C
72h
14mm
87%
3/1
1968
+11.3 C
65h
87mm
86%
25/10
1967
+14.4 C
122h
83mm
80%
+17.1 C
206h
69mm
69%
+17.6°C
242h
12mm
70%
1967
26/6
10/6
I
II
III
IV
57 days
47 days
62 days
121 days
21
21
21
18
AUTUMN
WINTER
SPRING
SUMMER
19/3
9/4
80%
41mm
128 h
3%
3mm
15h
PRIL
CH
JU

Fig. 2.1

All samples were fasting samples Food was not accessabl to the animals after 5 p m. the day before sampling Samples from man were taken before breakfast

I general each sampl consisted of 6 male and 6 female of each species Some tests wer repeated second year these will be mentioned i the specific section where they appear

The samples wer taken as follows

Season I

Fall period: Some of the hedgehogs are just beginning to enter hibernation

October 25 November 14 between 12 00 and 13 00 hours

Season II

Winter period: All hedgehogs are hibernati g

January 10 January 31 between 13 30 and 14 30 hours

Season III

Spring period: Most hedgehogs have completed thei final arousal from hibernation

March 19 April 9 between 11 00 and 12 00 hours

Season IV

Summer period: All hedgehogs are active

June 10 June 26 between 08 30 and 09 30 hours.

Figure 2 1 shows the temporal elationship between these periods and the changes in meteorologi al conditions It tarts 1/7 1967 and ends 30/6 1968 In the outermost ircl is the month i the next ircl i the average ambient temperature i degrees C lcius next number of hours of sunlight next precipitation in millimeters next the average per cent relative humidity The next two ircles represent the time of sampling The next ircle represent the duration of the sampling period i days and the time between each period I the innermost ircl the four seasons are presented and i the very enter th time i hours between sunset and sunrise is graphically presented by the length of the radius from the enter The data is taken from the 1st and the 15th day of each month The distance from the enter to the innermost ircl represents 12 hours. The meteorological data is from the Swedish weather bureau (Statens meteorologiska och hydrologiska Institut)

Note that the time period between the Summer and Fall samples i about twi as long as the others (120 days)

Anesthesia: All animals with the exception of the hibernating hedgehogs during season II were anesthetized with 3 3 ml/kg of chloral hydrate-urethane solution (18 75 g urethane 2 25 g chlor l hydrat steril water qs to 100 ml)

Fig 2 1 See text for explanation

General measurements: Befor anesthesia each animal was weighed to the nearest gram and superficial abdominal body temperatur was measured to the nearest 0 1° C using thermocouple (Ellab Copenhagen) These data plus the date time of day and sex of the animal were recorded

The lectrocardiogram (standard leads I II and III) was recorded on ll animal and human subjects using direct writing ink jet recorder (Minograph Elema-Schönander Solna tockholm) Blörck and Johansson (1955)

Blood samples: Ten milliliters of venous blood was drawn from each unanesthetized human subject using a cannul without syringe allowing the blood to flow di ectly into the sampl tube This technique was used to minimize hemolysis and the consequent changes i serum ion ratios Thi was the only sampl taken from the human subjects i thi study

After anesthesi the thoracic cavity of the hedgehog or guinea-pig was opened A hypodermi needl size no 1 8 was placed in th aorti arch of the hedgehog or the left ventri l i the guinea-pig From this needl the following samples were drawn

1) Whol blood in gas tight heparinized syringe was taken for the determination of PaO_2 $PaCO_2$ pH and standard bicarbonate
2) Whol blood was taken by micropipette for enzymatic blood glucose determination and for hemoglobi determination
3) As much as possibl of the blood remai ing i the animal was the removed by syringe The blood was allowed to lot Thi was then centrifuged at 1600 rpm for 10 minutes The resulting supernatant was centrifuged at 1500 rpm for 5 minutes.

   The serum so obt ined was divided for the following analyses:

A) Sodium potassium cal ium and chlorid
B) Urea Creati ine Total protei
C) Insuli
D) Lacti dehydrogenase (LDH) Glutamic oxalacetic transaminase (GOT) Glutami pyru- i transaminase (GPT)
E) Total fat Triglycerides Fre fatty acids Cholesterol Lipoprotei lectrophoresis

All of the above parameters with the exception of the blood gas measurements were also measured on the venous blood from the human subjects

Tissue samples

Heart: As much as possibl of the great vessel pericardium and fat were removed The whol heart was then rinsed in saline blotted and weighed to the nearest 0 001 gram on Mettler singl pan analytical balance

About 60 mg of the left ventricl from the apex was weighed and placed i small tube frozen on dry i and stored at 70° C for analysis of activity of 3 enzymes i catecholami catabolism (Monoamine oxidase (MAO) catecholamine-O-methyltransferase (COMT) and phenylethanolamin (PNMT))

Another small piece of left ventri le from the apex and small piece from the right atrium near the SA node wer placed on small labelled pieces of typewriter paper and frozen i propane propylene mi ture and stored i liquid nitrogen These samples wer studied by fluorescenc microscopy to visualize patterns of adrenergic innervation

A small piec of left ventricl from the apex was weighed and minced with scissors and placed in perchloric acid for determination of epinephrine and norepinephrine concentration

About 1 gram from the middle of the heart was weighed and placed in small tube frozen on dry i stored at 20° C and saved for glycogen determination.

A small piec of heart tissue was frozen i dry ic stored at 20° C and saved for determination of fibrinolytic activity

The est of the heart mainly from the base was weighed placed in small tube frozen on dry ice and stored at 20 C for studies on lactic dehydrogenase (LDH) mali dehydrogenase (MDH) alpha-glycerol phosphat dehydrogenase (α-GPDH) and glycose-6-phosphat dehydrogenase (G-6-PDH)

Lung: A small piece of lung tissue was saved for determination of fibrinolyti activities as above

Adrenals: The adrenals wer removed and leaned of excess blood and fat and weighed to the nearest 0 0001 gram

The left adrenal was weighed placed i small tube frozen on dry ice and stored t 70° for analyses of the three enzymes of catecholamine catabolism (MAO COMT PNMT)

The ight adrenal was weighed minced with scissors and placed i tube of perchloric acid for determination of epinephrine and norepinephrine concentration

Kidney: A small piec of kidney was saved for analyses of fibrinolyti activity as above

Liver: The liver was cleaned rinsed i saline blotted and weighed to the nearest 0 001 gram.

Approximately one gram of tissue was weighed placed i small tube and frozen on dry ice This was stored at 20 C for studies on LDH MDH α-GPDH and G-6-PDH

The est of the liver was weighed placed i tube frozen on dry ic and stored at 20° C for glycogen determination

Pancreas: The pancreas was leaned blotted and weighed t the nearest 0 001 gram It was then frozen on dry i and stored at 70° C for analysis of insuli content

Spleen: The spleen was leaned insed i saline blotted and weighed to the nearest 0 001 gram. It was then discarded.

Testes: The accessory tissue was removed and the testes were blotted and weighed to the nearest 0 001 gram They were then discarded

Vas deferens: A small piece of the vas deferens was prepared for fluorescenc microscopy as above

Ovaries: The ovaries were cleaned blotted and weighed to the nearest 0 001 gram and discarded

Uterus: The uterus was cleaned rinsed i saline blotted and weighed to the nearest 0 001 gram

A small sampl from one of the horns near the ervix was prepared for fluorescence microscopy as above

Thyroid gland: A small piec of the thyroid gland was prepared for fluorescence microscopy as above

Pituitary: The pituitary gland was removed and prepared for fluorescenc microscopy as above

White fat: Abdominal subcutaneous fat was removed Whit fat weighing 0 5 g or less was placed in small tube frozen on dry ice and stored at 20° C for gas chromatographi analysis

About one gram of whit fat was weighed placed in tube frozen on dry ice and stored at 20 C for analysis of LDH MDH α-GPDH and G-6-PDH

Brown fat: The superfi ial lobe of the i terscapular brown f t was dissected out from the hedgehog (It was not possible to get reasonabl sampl from guinea-pigs) This was blotted on filter paper and weighed to the nearest 0 001 gram

Brown f t weighing 0.5 g or less was placed i a small tube frozen on dry ice and stored at 20 C for gas chromatographi analysi

About one gram of brown f t was weighed placed i tube frozen on dry ice and stored at 20 C for analysis of LDH MDH G-PDH and G-6-PDH

A small piece of i terscapular brown f t was then prepared for fluorescence microscopy as above

Data handling and statisti l methods

Wherever the results from the studies reported i thi volume yi lded digital esults amenable to statistical analysi they wer analyzed together with the results from other tests by means f a computer program "Season Thi program modified from units f the IBM Scientifi Subroutine Package was run on the Cleveland Stat University IBM 360/40 computer The program llowed selection of data on the basi of th other parameters of that sampl _g season sex and non zero value The output gave mean standard deviation standard error of the mean maximum minimum sum sum of squares number of samples for each variabl with each season and students "t "F values and degrees of freedom for comparison of ignificanc of differenc between season sex and species

Chapte 3

Body d Org W ights Body T mp tu

by

Jerome B Senturi and Bengt W Johansson

Methods and materials

These are described I Chapter 2 General Methods and Material

Results

The results are given I Figures 3 1 to 3 12

Body Temperatur (Fig 3 1) The mean body temperatur of the hedgehogs was lowest I the hibernating group (Season II) and slightly depressed I the pre and post hibernating season groups (I and III) this is accounted for by those animals which wer just entering hibernation I Season I and those just exiting hibernation I Season III

The guinea-pig shows constant body temperature year round with light hi t of hypotherml I winter

Body Weight (Fig 3 2) The hedgehog is heaviest I the fall loses weight during the hiber nation and arousal seasons and regains weight during the summer The differences ar not statisti-cally ignificant however

The gui ea-pig shows different pattern It I heaviest I the winter and lightest during the summer

Heart Weight (Fig 3 3) I both species the same pattern is seen The heart I heaviest I the winter and lightest I the summer

Liver Weight (Fig 3 4) The liver is heaviest I the summer I the hedgehog and lightest I the winter I the guinea-pig however the opposite I true; the liver I heaviest I the winter and lightest I summer The liver weight seems to parall l body weight

Spleen W ight (Fig 3 5) I th hedgehog the spleen I heaviest in summer and lightest I winter I the guinea-pig there appears to be no seasonal change

Weight of Pancreas (Fig 3 6) I the hedgehog the pancreas I smallest I winter and largest I summer There is no seasonal hange apparent I the guinea-pig

Weight of Interscapular Brown Fat (Fig 3 7) I the hedgehog the brown fat I heaviest I the F ll and decreases through the summer No samples could be obtained from the guinea-pig

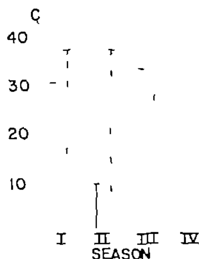


Fig. 31

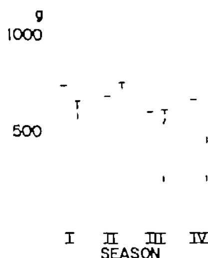


Fig. 32

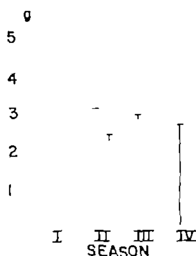


Fig. 33

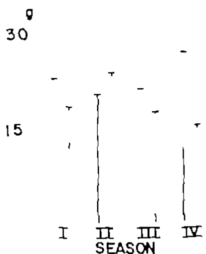


Fig. 34

W ight of Adrenal (Figs 3 8 and 3 9) Both individually (Fig 3 8) and combined (Fig 3 9) the adrenal of the hedgehog appear to be smallest in the wi ter I the guinea-pig the adrenals appear to be heaviest I the winter These differences only appear ignificant I the singl adrenal weights (Fig 3 8)

Weight of Testes (Fig 3 10) The weight of the testes changes strikingly with seasons I the hedgehog lowest I the fall and highest I the spring and summer No signifi ant weight change is seen I the case of the guinea-pig

Weight of Ovaries (Fig 3 11) Another pattern I observed for the ovaries which I the hedgehog are heaviest in the f ll whil I the guinea-pig the highest values are found in the winter but the differences are small

Weight of Uterus (Fig 3 12) In the hedgehog the lowest values are found I the wi ter but I both animals during all seasons the changes are small

Discussion and conclusions

The changes I body temperature seen I the hedgehog learly demarcat the seasonal om-ponents of the hibernation cycl The body weight pattern I the hedgehog demonstrates two of the features of the hibernation cycl the weight gain in the f ll represents "preparation for the coming hibernation season the loss of weight during the hibernation and arousal season demonstrates the weight loss under the reduced metaboli stat of hibernation with its concomitant essation of food intake and the high utilization of energy stores during arousal Thi pattern of weight hange agrees well with the work of thers (Bärck et l 1956b

Fig 3 1 Body temperature in degrees C lsius Season efers to the pr hibernating (I) hiber nating (II) posthibernating (III) and active non-hibernating (IV) phase See further Fig 2 1 I Chapter 2 General Methods and Material The solid line refers to hedgehogs and the interrupted line to gui ea-pigs The number of denot the ig-ificanc level according to the "t test one means 0 05> P> 0 01 and two P< 0 01 E refers to the hedgehog (Erinaceus europaeus) and C p the guinea-pig (Cavia porcellus) 1 indicates the differenc between seasons I and II 2 between I and III 3 between I and IV 4 between II and III 5 between II and IV and 6 between III and IV

Fig 3 2 Body weight See Fig 3 1 for legend

Fig 3 3 Heart weight See Fig 3 1 for legend

Fig 3 4 Liver weight See Fig 3 1 for legend

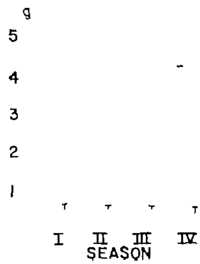


Fig. 3.5

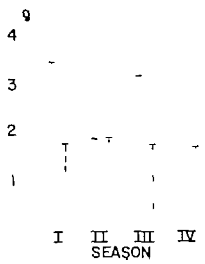


Fig 3.6

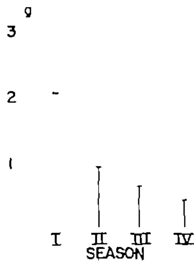


Fig 3.7

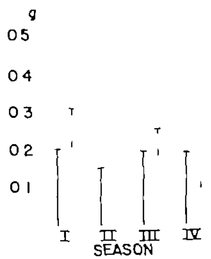


Fig. 3.8

Pengelley and Kelly 1967) I comparison it should be noted that the amplitud of the w ight cycl of the hedgehog is much less than that seen with the rodent hibernators

The pattern of weight change in the guinea-pig is I sharp contrast to that I the hedgehog Here the animal tends to fatten I the winter months due in part to increased intake of food The animal's metaboli rate is greater I old because as non-hibernating homeotherm it must generate more heat to eplace that lost to the environment Although the data does not directly show it these animals had onsiderably mor subcutaneous fat in the winter season and wer thinnest I the summer season The adaptive advantage of increased fat as insulating material for the maintenanc of body temperature is lear

I the guinea-pig the changes in the weight of the heart follow the same pattern as body weight hanges. The hedgehog, however shows small increase I winter which might represent an adaptation of the heart muscl tissue to the additional work performed during arousals.

The weight of the liver shows seasonal hanges I both species which reflect their changes in body weight

The weight of the spleen in the hedgehog decreased in the winter This is I contrast t th work of Soivio (1967) who demonstrated an increase I relative size of the spleen at the beginning of the hibernation season and that the spleen continued large during hibernation The observations on the spleen I the literature ar divergent and one important reason for thi is species differences Another factor is the time after the onset of arousal that the spleen I studied According to Lidi ker and Davis (1955) there I considerabl drop I the rat! spleen weight/body weight as soon as 5 minutes after the animal was taken out of the refrigerator It may well be thi time factor whi h explains the differences between our results and those reported by Soivi (1967) because weighing procedure the ecording of an lectrocardiogram and the collecting of blood samples were our I itial procedures and could cover up to ten minutes

The changes in the pancreas I the hedgehog are probably due t the lack of food intake and the subsequent atrophy of the exocrine pancreas c ll It I difficult to comment on how this change I weight relates to the Beta lls of the pancreas No change is seen in the guinea-pig pancreas weight

The brown fat has been implicated as major sourc of energy for spontaneous arousal I *hibernating animal (Smith and Hock 1963 Smalley and Dryer 1967 Hayward and Lyman* 1967) Our data shows decrease in the weight of brown f t from the interscapular region of the hedgehog through the winter and spring to the summer The summer animals appear to

Fig 3.5 Spleen weight See Fig 3 1 for legend

Fig. 3 6 Pancreas weight See Fig 3 1 for legend

Fig 3 7 Weight of interscapular brown fat See Fig. 3 1 for legend

Fig 3 8 W ight of left adrenal See Fig 3 1 for legend

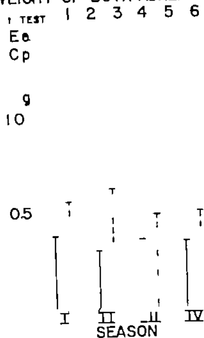


Fig. 3.9

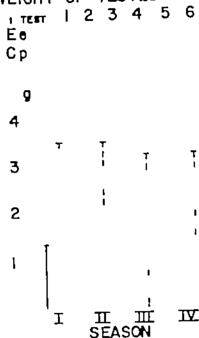


Fig. 3.10

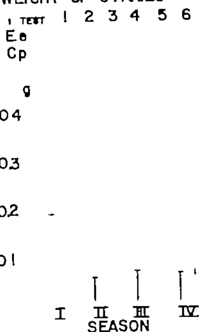


Fig. 3.11



Fig. 3.12

have the smallest amount of brown adipose tissue while the greatest weight of interscapular brown fat i found in the fall season which is indicative of preparation for the metabolic changes associated with hibernation and arousal The literature on the effect of stress on this tissue has been discussed i previous paper (Johansson 1959)

The adrenal in the hedgehog are smallest in the winter The differences appear significant when one compares left adrenal rather than the pair One suspects that the significanc of this is that the hedgehog i less stressed i the winter and the production of cortical steroids is lower whil in the guinea-pig the adrenals ar largest i winter and the animal is probably undergoing the stress of cold acclimation

The testes ar clearly smaller i the hedgehog in the fall and winter months and the increase is such that i the spring season the testes are at their full weight The gui ea-pig hows no sign of such seasonal breeding cycl

I the hedgehog the uterus is smallest in the winter and largest i the summe and fall but the statistical significanc is marginal Thi lso reflects a seasonal breeding cycl in the hedgehog The guinea-pig shows cycl in uterine weight but it is not statistically significant

No clearcut cycl i weight of ovaries can be seen i either species but the hedgehog does appear to have heavier ovaries i the fall

Fig 3 9 Weight of both adrenals See Fig 3 1 for legend

Fig 3 10 Testes weight See Fig 3 1 for legend

Fig 3 11 Weight of ovaries See Fig 3 1 for legend

Fig 3 12 Uterus weight See Fig 3 1 for legend

Chapter 4

El ctrocardiog m

by

Barbro Eklund Jerome B Senturia and Bengt W Johansson

Studies of the seasonal variations of the lectrocardiogram (ECG) I animal are scanty Comparisons betwe the pronounced bradycardia during hibernation and its reverse when the hibernator I not I the hibernating state is an exception (Johansson 1967)

Methods and materials

These are described in Chapter 2 General Methods and Materials

I hedgehogs electrodes were placed I the left and right scapular and the left gluteal regions as described previously (Blörck and Johansson 1955) The ECG in the guinea-pig was ecorded as the three standard leads I II and III with the aid of needl lectrodes with the animal lying with its back upwards The same three standard leads were ecorded with conventional technique and also with di ect writing I k jet recorder (Elema Mingograph Stockholm-Solna Sweden) I man being I supine position

Conventional durations I the ECG wer obt ined including the Q-T interval I per ent of the R-R interval to ompensate for the Q-T changes produced by hanges I heart ate (Blörck and Johansson 1955)

Results

Sinus arrhythmi occured to ertain extent I ll three species but no major arrhythmias wer found P waves were not detectabl I the ECG from hibernating hedgehogs these are often difficult to find and need not impli ate any arrhythmi The results ar presented I Tables 4 I 4 II and 4 III

Hedgehog (Tabl 4 I) The pronounced difference I heart rat between hibernating animals season II and non-hibernating animals seasons I III and IV I well established The differences between seasons I III and IV wer not significant There was a tendency to shorter ECG I tervals in animals from season I especially when ompared to season III and most pronounced for P-R ($0.01 < P < 0.05$) and Q-T (P 0.001) The faster heart rate I season III might contribute

Table 4.I

	Heart rate in beats/min			R-R in sec			P-R in sec			QRS in sec			Q-T in sec			Q-T in % of R-R		
	M	n	SD	M	n	SD	M	n	SD	M	n	SD	M	n	SD	M	n	SD
Season I	249.3	3	21.3	0.250	3	0.025	0.100	3	.005	0.104	3	0.016	0.136	[illegible]	0.019	53.9	3	7.3
Season II	13	13	1	0.446	1	2.380				0.133	13	0.028	.330	11	0.000	.3	1	3
Season III	[illegible]	10	49.3	0.234	10	.046	0.099	10	0.009	0.033	10	0.008	0.140	10	0.000	46.9	10	0.2
Season IV	256.5	13	27.7	0.247	12	-0.063	0.081	10	0.000	0.080	11	.000	0.114		0.000	47.4	11	10.0

Time components of the ECG in the hedgehog. Season I fall Season II winter Season III spring, Season IV summer
See further chapter 2.

Table 4.II

	Heart rate in beats/min			R-R in sec			P-R in sec			QRS in sec			Q-T in sec			Q-T in % of R-R		
	M	n	SD	M	n	SD	M	n	SD	M	n	SD	M	n	SD	M	n	SD
Season I	280.7	14	37.4	0.245	14	.039	.080	14	0.009	.047	14	.000	0.131	14	0.030	54.3	14	7.7
Season II	279	12	30.3	.270	12	.033	0.085	12	0.01	0.033	12	0.000	0.13	12	0.014	52.3	12	4.2
Season III	269.8	12	31	0.230	1	-0.044	0.050	12	.050	0.030		.000	0.116	12	.033	50.8	12	6.3
Season IV	271	12	22	0.275	12	0.041	0.086	3	.000	0.030	12	.008	.130	12	-0.090	47.0	3	6.0

Time components of the ECG in the guinea-pig.
See further legend table 4.I.

Table 4.III

	Heart rate beats/min			R-R in sec			P-R in sec			QRS in sec			Q-T in sec			Q-T in % of R-R		
	M	n	SD	M	n	SD	M	n	SD	M	n	SD	M	n	SD	M	n	SD
Season I	73.3	13	10.	0.843	13	.134	0.133	13	.007	.080	13	0.000	0.334	13	.014	43	13	
Season II	75	12		0.840	12	.147	0.103	13	0.023	.073	12	0.000	0.330	1	0.030	37	3	.7
Season III	40	13	11.3	.900	13	.140	0.135	13	0.030	.073	13	0.008	.340	13	.030	30.3	11	
Season IV	44	13		0.919	13	0.171	0.142	13	.036	.077	13	-0.000	0.342	1	0.032	37	13	3.4

Time components of the ECG in man.
See further legend table 4.I

to these differences but also Q-T in per cent of R-R was shorter in season III 45 9 compared to season I 52 0 lthough not significantly so (P > 0 05) All components of the ECG from hibernating animals season II were markedly prolonged and the R-R interval to such an extent that Q-T i per ent of R-R was six times less than in non-hibernating animals despite an absolut prolongation of Q-T

Guinea-pig (Table 4,II) The heart ate i the guinea-pig was slower during season IV summer mean 221 9 beats per minut than during season II winter mean 279 9 beats per minute (P 0 001), The values for seasons I and III f ll betwee these two extremes Only the difference between seasons III and IV was significant (0 05 P 0 01)

No major differences were found for the P-R or QRS durations. The Q-T duration was 0 11 seconds in season II against 0 13 i season IV which is in accordanc with the higher heart rate i season II The Q-T in per cent of R-R remained mai ly unchanged with the seasons and only the difference between seasons I and IV was ignifi ant (0 05 P 0 01)

Man (Tabl 4,III) The highest heart rat was obtained i season I 72 8 beats per minute and the lowest i season IV 66 4 thi differenc being significant (0 05 > P 0 01) The P-R and QRS durations did not vary ignificantly The Q-T duration i per ent of R-R differed between seasons I and IV (0 05 P 0 01)

Discussion

The pronounced ffect of temperatur on the ECG has been pointed out previously (Biörck and Johansson 1955) Many hibernators are characterized by an extremely short S-T segment (Johansson 1967) The possibl importance of thi finding for the resistanc to ventricular fibrillation (Johansson 1963) i as yet not settled

During arousal the hibernators sympatheti system i driven at very high rat (see Johansson 1967 for review) It was therefor of interest that the heart rates dur and especi lly i season III exceeded those previously eported during arousal (Johansson 1955) The additional effect of anesthesi was considerabl for the maximal heart rat during arousal 181 beats/min was exceeded by the average value obtained during season III 277 beats/mi

The higher heart rat i the gui ea-pigs during season II winter i omparison to season IV summer might well be an adaptation to the lower ambient temperature during winter I man ther is progressive drop i heart rat from season I fall to season IV summer Despite the fact that all the human subjects wer well acquainted with the hospital milieu it may well be that light nervousness explains the initi l light tachycardi during season I fall

Chapter 5

Blo d Ion

by

Jerome B Senturia Barbro Eklund and Bengt W Johansson

Fisher and Manery (1967) point out in thei review on ion and water balances the lack of consistency between investigations of the hanges in blood ions as function of stage in the hibernation cycle We hope that the results presented here will help to clarify rather than confound an area already replete with inconsistencies

Methods and materials

The general methods and materials are outlined in Chapter 2 Sodium potassium and cal ium were determined using an Eppendorff flame photometer Chloride was determined using Technicon Autoanalyzer [1] using modified method of Schales and Schales (1941) These analyses were run together with the routine analyses done by the Clinical hemistry laboratory of Malmö General Hospital

Results

Figures 5 1 5 4 how the data obtained i this phase of the study

Sodium (Fig 5 1) I man sodium i very slightly higher i f ll than summer and thi small differenc i statist ally ignificant

I the hedgehog the differences are agai small but the statisti al significance mor pronounced Sodium is seen to be highest i summer and decreases through to the spring

I the guinea-pig the pattern is mor striking with the lowest values in winter and spring

Potassium (Fig 5 2) I man we see significantly higher potassium value i fall decreasing to its lowest value i summer

I the hedgehog there i decrease i potassium during the winter

I the guinea-pig the seasonal hange i of smaller magnitude and is i different direction

1) Technicon Instruments Company Chauncey N J

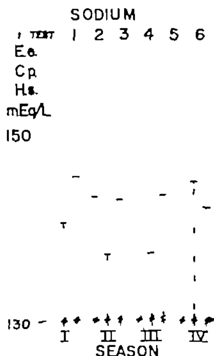


Fig. 5.1

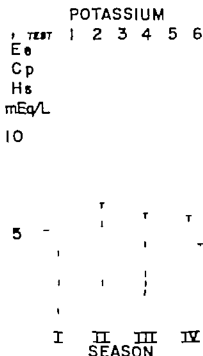


Fig. 5.2

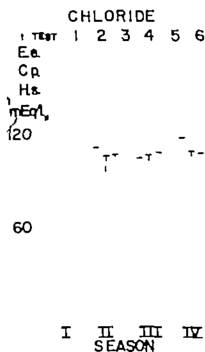


Fig. 5.3

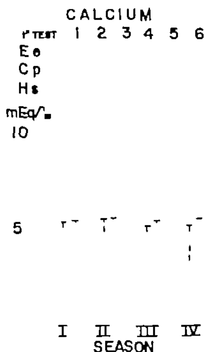


Fig. 5.4

Chloride (Fig. 5 3) The chloride values for all three species seem to follow the hanges I sodium.

Calcium (Fig 5 4) I man we see a slightly lower value for serum calcium in the fall

I the hedgehog we find the serum calcium highest I wi ter

In the guinea-pig we find a similar pattern to that seen I the hedgehog but of lower ampli tudes

Discussion and conclusions

Other workers report no seasonal change in the serum sodium I hibernators (Pengelley and Kelly 1967 Edwards and Munday 1969b) The light decrease in sodium seen I the hedgehog during the hibernation season is paralleled by the decrease I potassium seen I thi work. The small changes in the guinea-pig suggest a dehydration during the summer and possibly the f ll It is difficult to interpret whether the change seen I man is physiologically significant

I man the seasonal change I potassium seems to follow that of sodium. I the hedgehog the decrease I potassium seen in the winter is I good agreement with the work of Christian (1961) who found differenc between hibernating and non-hibernating woodchucks and with previous studies in the hedgehog (Bärck <u>et al</u> 1956b)

Thi data however is I sharp contrast with that of Eliassen (1963) who found about 4 Meq/L I August and September and about 7 Meq/L I December January and February I the hedgehog There was unfortunately no tatistical comparison made Edwards and Munday (1969b) found no difference I plasma potassium between hibernating and non-hibernating hedgehogs Pengelley and Kelley (1967) made K^+ determinations on active and hibernating

Fig 5 1 Serum sodium I mEq/L Season refers to the prehibernating (I) hibernating (II) posthibernating (III) and active non-hibernating (IV) phase See further Fig 2 1 In Chapter 2 General Methods and Materi l The solid line efers to hedgehogs the interrupted line to guinea-pigs and the dotted line to homo The number of denote the significanc level according to the "t test one means 0 05 P 0 01 and two P 0 01 E refers to the hedgehog (Erinaceus europaeus) C p to the guinea-pig (Cavi porcellus) nd H to man (Homo sapiens) 1 indicates the differenc between season I and II 2 between I and III 3 between I and IV 4 between II and III 5 between II and IV and 6 between III and IV Indi idual values for the human subjects are given I the Appendix tabl

Fig 5 2 Serum potassium See Fig 5 1 for legend

Fig. 5.3 Serum hloride See Fig 5 1 for legend

Fig. 5 4 Serum lcium. See Fig. 5 1 for legend

individual ground squirrel (Citellus lateralis) They found that the active animals had serum potassium of 9 6±0 70 Meq/L and when the same animals were in hibernation they had a serum potassium value of 6 0±0 64 Meq/L They suggest that there are technical reasons such as lysis of red cells after sampling which have led to the differences betwee result obtained by others The physiological ignificance of altered ion ratios and permeability has been suggested by Senturi t al (1970) and Pengelley and Kelley (1967) If the hibernator is t have the heart and nervous system function at lower temperatures then membrane potential differences must be maint ined in spite of the lowered activity Edwards and Munday (1969b) have shown that many of the ion ratios between the tissues and the plasma remain constant between awake and hibernating hedgehogs In their study they did not examine nervous and heart tissue for changes in sodium and potassium ratios lso their data does show changes i the sodium rati for two of the three muscl tissues tested

Since temperature i factor in the membrane potential equation some compensation must be made in either the permeability or in the ratios of ions in order for the membrane potential to be effective at lower body temperatures

$$E_m \quad \frac{RT}{F} \ln \left(\frac{P_{K^+}(K^+)_{out} \quad P_{Na^+}(Na^+)_{out} \quad P_{Cl^-}(Cl^-)_{in}}{P_{K^+}(K^+)_{i} \quad P_{Na^+}(Na^+)_{in} \quad P_{Cl^-}(Cl^-)_{out}} \right)$$

The Goldman equation where:

E_m = Membrane potential

R = Universal Gas Constant

F = Faraday

T = Absolut Temperature

P_k = Permeability of membrane to potassium

$(K^+)i$ = Concentration of potassium insid the membrane

$(K^+)out$ = Concentration of potassium outside the membrane

The permeabilities and conc ntrations of other ions are denoted i similar manner

Marshall and Willi (1962) showed a lear diff rence between the auricles of rabbits (nonhibernators) and ground squirrel (hibernators) in their ability to maintain membrane potential at low temperature

In the guinea-pig the seasonal hange i potassium is of smaller magnitud and opposite di ection of that of the hedgehog It is not clear why the guinea-pig has higher serum potassium in the winter, but there may be greater tendency of wi ter red blood cell to lyse

The chloride values for all three species seem to follow the changes i sodium

It is difficult to ascribe physiological significance to the slightly lower serum al lum value seen I man I the fall season

I the hedgehog the serum calcium I highest in the winter Thi I in good agreement with Raths (1962) and Edwards and Munday (1969b) and with earlier studies from this laboratory (Biörck et al 1956b) This rise in serum calcium is possibly caused by the hyperactivity of the parathyroids and the subsequent decalcification of bones and teeth seen in hiber nation (Kayser et al 1964) Thi change may lso be due to changes I calcitonin activity (Nunez et al 1967 Gershon and Nunez 1970)

I the guinea-pig a lower amplitude hange of similar pattern occurs It I possibl that the guinea-pig lso has some sort of seasonal change in parathyroid activity but thi has not yet been examined

Chapter 6

Blood G ses

by

Bengt W Johansson and Jerome B Senturia

The ability of hibernators to tolerat anoxia (Biörck and Johansson 1957) prompted study on the blood gases including the arterial oxygen (PaO_2) and carbon dioxide ($PaCO_2$) tension pH and standard bicarbonat in hedgehogs and guinea-pigs

Methods and materials

These are partly described i Chapter 2 General Methods and Materi ls The first arterial sample drawn was used for blood gas analysis to minimize the effect of hypoxi PaO_2 was measured with modified Clark electrode (Clark 1956) and $PaCO_2$ according to Severinghaus and Bradley (1958) pH was measured with conventional electrode and standard bicarbonate according to Jørgensen and Astrup (1957) All samples were analyzed as soon as possibl at 37° C These analyses were run by the Clinical physiology laboratory of Malmö General Hospital

Results

The PaO_2 values were low throughout especi lly i the guinea-pigs (Fig 6 1) but seasonal differences did occur with higher values during summer 25 mm Hg than during f ll ($P < 0.01$) *The winter and spring values wer i between and did not differ from each other* The hedgehog values wer i general higher than those for the guinea-pig The winter values were highest in the hedgehog; the summer values were higher than those from f ll and spring although the differences were not significant

The $PaCO_2$ values (Fig 6 2) did not vary much i the guinea-pig whil hedgehogs showed very high value i winter 121 mm Hg as against half thi value during the other seasons The $PaCO_2$ values wer higher throughout i hedgehogs when compared to the guinea-pigs

The pH (Fig 6 3) values differed between species hedgehogs having lower values throughout than guinea-pigs The guinea-pig summer values were lowest 7 23 and the wi ter values were highest 7 29 ($P < 0.05$) The much lower winter value i hedgehogs

6 90 differed significantly from the values obtained during the other seasons (P 0,01)

Standard bicarbonat values were on the same level I both species (Fig 6 4) The higher winter values I the guinea-pigs differed significantly from those obtained during the other seasons and so was the case in the hedgehogs

Discussion

The PaO_2 values are low I both the guinea-pigs and the hedgehogs from all the seasons This is due both to the deteriorated respiration which developed in some of the animal as result of the anesthesia and the ineffective respiration during the time the thorax was open These two factors did not differ between the species and the seasons and yet the guinea-pig values were lower This is probably due to a mor intensive metabolism I guinea-pigs Inc the body weight of these animals I lower than that of hedgehogs The greater tolerance to hypoxia I hibernators even when in the non-hibernating stat when compared to non-hibernators (Biörck et al 1956a) is probably due to a functional adaptation at the tissue level and cannot be attributed to a systemic capacity for maintaining higher blood PO_2 during hypoxia (Burlington et al 1969) The high PaO_2 in the hibernating hedgehogs (66 mm Hg) I explained by the slower metabolism with concomitant decreased oxygen onsumption during hibernation Mor remarkabl are the higher PaO_2 values during the summer when compared to the fall; thi was most conspicuous I the guinea-pigs Thi might be partly explained by higher oxygen onsumption during the fall as an initial phase of the adaptation to a lower ambient temperature; this higher oxygen need I the tissues will result I lower PaO_2 provided the time of hypoxi is the same

The high $PaCO_2$ values I the hibernating animal onfirm previous reports Dubol (1896) even suggested that the inc ease I carbon dioxid ontent of the blood may be the ause of hibernation (autonarcosis) A high carbon dioxide content during hibernation was found by many authors I several animal species (Kayser 1961) It has bee shown by Siggaard Andersen and Egsbaek (1962) in hibernating bats that blood samples measured both at 8 C and 38 C differed the 8 C values yielding pH of 7 67 and $PaCO_2$ of 18 mm Hg; corresponding values for 38 C were 7 26 and 63 mm Hg espectively Thi differenc I $PaCO_2$ I less than the values we obtained I the hedgehogs I wi ter experiments I hibernating hedgehogs at 5° C pH value of 7 37 and $PaCO_2$ of 55 mm Hg was obtained orresponding values for the samples run at 37° C were 6 90 and 100 mm Hg (Johansson and Svanberg, unpublished observations)

It is lso noteworthy that the $PaCO_2$ values wer higher and the pH values lower I the hedgehogs when ompared to the guinea-pigs throughout the seasons Thi was so lso during the summer when the mean body temperatur was 35° C I the hedgehogs and 38 C I the guinea-pigs However the standard bi arbonate I e the bicarbonat concentration measured

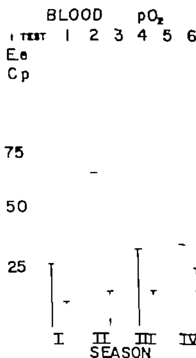


Fig. 6.1

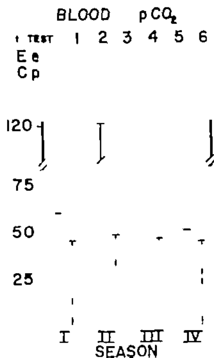


Fig. 6.2

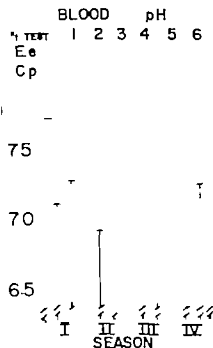


Fig. 6.3

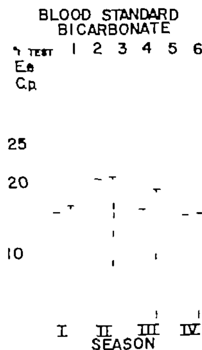


Fig. 6.4

when PCO_2 has been adjusted to 40 mm Hg was of the same magnitude in both species and I both significantly higher values were found during the winter than during the other seasons.

When comparing these observations I hedgehogs to those reported by Lyman and Hastings (1951) in golden hamsters and ground squirrels there is an agreement I the high PCO_2 values in the non-hibernating animal However our pH values in the hedgehogs ar lower and contrary to these authors we find an increase of the PCO_2 and a further decrease of the pH in the hibernating hedgehogs Ther ar some methodological differences which might contribute to explain these different results Lyman and Hastings (1951) determined plasma pH t 5° C I hibernating animals Furthermor they used the Henderson-Hasselbalch equation to calculat the PCO_2 Twente and Twente (1964) reported lower PCO_2 values I hibernating golden-mantled ground squirrels than we found in hedgehogs Species differences might contribute to the observed differences

Fig 6 1 Arterial blood oxygen tension (pO_2) The seasons refer to the prehibernating (I) hibernating (II) posthibernating (III) and active non-hibernating (IV) phase See further Fig. 2 1 I Chapter 2 General Methods and Materials The solid line refers to hedgehogs and the interrupted line to guinea-pigs The number of denote the ignificance level according to the "t test one means 0 05 P 0 01 and two P < 0 01 E. refers t the hedgehog (*Erinaceus europaeus*) and C p to the guinea-pig (*Cavia porcellus*) 1 indicates the difference between season I and II 2 between I and III 3 between I and IV 4 between II and III 5 between II and IV and 6 between III and IV

Fig. 6 2 Arterial blood carbon dioxide tension (pCO_2) See Fig 6 1 for legend

Fig 6 3 Arteri 1 blood pH See Fig 6 1 for legend

Fig. 6 4 Arteri 1 blood standard bicarbonate See Fig 6 1 for legend

Chapter 7

Nitrogen M tab lism S m V riable

by

Bengt W Johansson and Jerome B Senturi

The small amounts of blood availabl precluded us from getting detailed aspects of nitrogen metabolism. Serum urea creatinine and protei were selected for loser study

Methods and materials

These are partly described I Chapter 2 General Methods and Materials Serum urea creatinine and prot i were measured with Technicon Autoanalyzer[1] using modification of the methods described by Skeggs (1957) F II and Wu (Astra 1947) and the biuret eaction (Astra 1947) These analyses were run together with the routine analyses done by the Clinical hemistry laboratory of Malmö General Hospital

Results

The serum protein content (Fig 7 I) was lowest in spring I the hedgehog but the differences wer not significant Similar but more pronounced tendencies wer obtained I the guinea-pigs

1) Techni on Instruments Company Chauncey N J

Fig 7 1 Serum protei i grams per 100 ml Season refers to the prehibernating (I) hibernating (II) posthibernating (III) nd active non-hibernating (IV) phase See further *Fig 2 1 In General Methods and Materi ls The solid line refers to hedgehogs the* interrupted line to guinea-pigs and th dotted line to homo the number of denote the significance level according to the "t test on means 0 05 P 0 01 and two P 0 01 E refers to the hedgehog (Erinaceus europaeus) C p to the guinea-pig (Cavi porc llus) and H to man (Homo sapiens) 1 indicates the dif ference between season I nd II 2 between I and III 3 between I and IV 4 between II and III 5 between II and IV and 6 between III and IV Individual values for the human subjects ar given I the Appendix tabl

Fig 7 2 Serum urea I mg per 100 ml See Fig 7 1 for legend

Fig 7 3 Serum creatinine I mg per 100 ml Se Fig 7 1 for legend

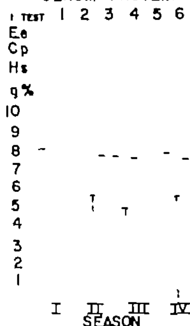


Fig. 71

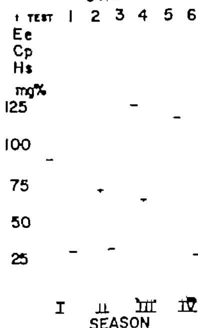


Fig. 72

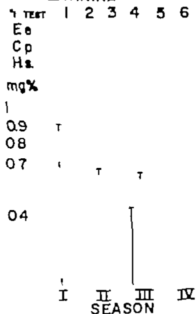


Fig. 73

these values were consistently lower than in hedgehogs and man A seasonal variation was also observed in man with highest values in f ll

Urea concentration (Fig 7 2) was consistently higher in hedgehogs than i man with the guinea-pig values i between The lowest values wer obtained i fall i the hedgehogs whil in the guinea-pigs the reverse was found No seasonal variations occurred i man.

Creatinine values (Fig 7.3) were higher i man than in the other two species Agai no seasonal variation was found in man whil i guinea-pigs the same pattern was found as for urea i hedgehogs the highest values were obtained i winter

Discussion

The absence of seasonal variations i serum urea and creati ine i man indicat a fairly constant protein intake and catabolism and good kidney function The finding of high urea values around 100 mg per ent i our hedgehogs i i conformanc with Kristoffersson's (1963) observations W lso observed as did Kristoffersson (1963) that the values from non-hibernating summer hedgehogs were lower than those from the hibernating animals lthough the difference was not statistically significant i our animals This difference was not as high as reported by Kristoffersson (1963) and this may be due to methodological variations because his hedgehogs were fed on high protei diet and had been fasting for one day prior to sampling of blood for urea analysi

Our fall values were significantly lower than our winter and spring values Since there i no reason to suspect any changes i the renal function the creatinine values were on the same level i f ll and summer it i probabl that the low values i f ll are due to an increased anabolic activity; this would fit i very well with preparation for hibernation A decreased intake of prot ins ould lso explai the low urea values Although no detailed analysi of the onsumed food was undertaken ther were no signs of hanges i the food intake and there were no systematic variations i the composition of the food given to the animals.

Our findings gree with other observations *i hedgehogs (Johansen 1964)* that blood creatinine and urea i lower prior to than during hibernation. Our observations in the guinea-pig showed everse pattern with higher urea values i fall and summer than i winter and spring The creatinine values followed this pattern

The guinea-pigs differed from the other species by having lower serum protein values around 5 gram per ent against values around 8 i hedgehog and man A seasonal variation was observed i the guinea-pigs with low values i spring whil i man low values were found lso i summer No statistically significant observations wer noted in the hedgehogs which is in agreement with previous reports from thi animal (Biörck et l 1956b) The analysi program i the present study did not include protein electrophoresis but our previous observation (Biörck et al 1956b) of decrease of the γ-globulins during hibernation has been onfirmed in hamsters (South and Jeffay 1958)

Chapter 8

Carbohyd ate Met bolism

by

Jan Thorell Bengt W Johansson and Jerome B Senturia

The seasonal changes in blood glucose that occur i hibernators with lowest values during winter (Kayser 1961) have suggested that blood glucose and its egulation plays central rol for the induction of the hibernating stat Thi has also been supported by reports of suc essful induction of hibernation by the administration of insuli (Laufberger 1924 Dworki and Finney 1927 Suomalainen 1938) I other laboratories however thi technique has not been successful (Kayser 1955 Johansson 1972b) T our knowledge the endogenous insuli in the hedgehog has not been studied befor I thi work attempts were mad to assay insuli i plasma and extracts of pancreas of this animal to determine if seasonal changes could be shown. The elation to the seasonal habits of thi animal to these changes was examined The variation of blood glucose during the year in hibernating and non-hibernating species was also studied

Methods and materials

Blood was collected from 48 hedgehogs grouped as described i General Methods and Material Chapter 2 Clotting was prevented by ethylenediaminet tracetic acid (EDTA) Plasma was stored at 20 until analyzed

Intravenous glucose i jection (1 g/kg body weight i 25% solution) was given to three non-hibernating animal in J ly and August under light general anesthesi with hloral hydrate-urethane Blood was ollected through an i travenous catheter in the right atrium at i ter val up to 1 hour I two additional animals aroused from hibernation i December the same procedure was performed except that continuous intravenous infusion with phentolamine 0 03 mg/kg body weight and minut was started immediately after the injection of glucose

Insuli was assayed by radi immunoassay using modification (Thorell 1968) of the method of Yalow and Berson (1960) Insuli was extracted from the pancreas by modification of the method of Romans (1954) The excised pancreas was stored dry at 20° until extracted The piece was homogenized and extracted cold for one hour with 2 5 ml per g of the following

solutions 92% ethanol (v/v) contai ing 8% (w/) Na Cl pH adjusted to 4 0 with concentrated HCl The mixture was entrifuged and the solid material was reextracted for one hour with the same volume of 75% ethanol containing 1 5 ml of conc HCl/100 ml The solid material was emoved by entrifugation and two extracts were pooled They wer diluted 1:3200 with 0 75 M sodium barbital buffer pH 8 6 containing 0 25% bovine serum lbumi for the immunoassay Glucose was determi ed by glucose oxidase method Tissue glycogen was determined according to the method of Rerup and Lundqvist (1967) and expressed as glycogen l per cent of wet tissue

Results

Blood glucose: I hedgehogs marked reduction of blood glucose occurred during the winter period decreasing to an average of 38 mg/100 ml This orresponds to decrease of 54% in comparison with the mea of the spring and autumn values I the summer period (compared to spring) decrease was found I general 32% (Fig 8 1)

I the referenc groups of men and guinea-pigs the only seasonal hanges found were decreases I blood glucose during the summer period I relation to the mean values for autumn and spring the drop was 13% in men and 31% I guinea-pigs The absolute values for man and the hedgehog were very imilar in f ll and spring

Insulin: I serum samples collected from hedgehogs given no treatment before blood was take no immunoreactive insuli effective I the assay system was found Thi result was unaffected by which season f the year the sampl was taken

After the administration of glucose intravenously to three non-hibernating hedgehogs the blood glucose eached 230-780 mg/100 ml As may be seen I tabl 8 I (first three animal) the ffect on plasma insuli was irregular but immunoreactive materi l was found I plasma from two of the three animal tested In one of them there was substanti l ise soon after i jection of glucose while I the other one the increase was not ecorded until 40 minutes after the injection of glucose

By pretreatment of the hedgehogs with phentolamine the administration of glucose evoked constant ise i plasma insuli (Table 8 1)

By assay of extract of the pancreas high concentrations of immunoreactive insulin were constantly found (Tabl 8 II) I the organs collected I November and January mean concentration of insuli I the pancreas was 156 and 173 mU/g respectively Thi differenc was not significant In the nimals wher the pancreas was collected I J ly the ontent of insuli was considerably higher the average being 762 mU/g Thi was signifi antly higher than I the other animal (P 0 001) As the organs were also larger I summer hedgehogs total extractabl insulin was lso ignificantly higher I summer

Glycogen: The heart and liver glycogen values ar higher throughout I the hedgehog than I the guinea-pig The highest values ar found I wi ter and the lowest I summer I the

Tabl 8 I

Date of test	Type of test		Before	10 ml	20	40
27/7	G	Insulin µU/ml	3	3	3	3
		Glucose mg/100 ml	122	330	300	264
13/8	G		3	36	40	40
			64	230	193	164
13/8	G		3	3	3	25
			112	527	339	298
15/1	G+P		3	6	25	150
			93	790	636	613
15/1	G+P		3	22	42	65
			7	417	304	291

Plasma insulin and blood glucose at various i tervals after glucose load in non-hibernating hedgehogs Two types of tests were performed: G = Glucose load 1 g/kg body weight intravenously G+P = The same glucose load and continuous infusion of phentolamine 0 03 mg/kg body weight and minute

Table 8.II

Date of collection	Number of animals	Pancreatic weight gram	Insulin mU/g of pancreas	Insulin mU I total pancreas	Significanc of differences i insulin/pancreatic weight i relation to seasonal group	
5/11	10	1 79^{+}0 65	156^{+} 20	250^{+} 71	0 05	16/1
					0 001	1/7
16/1	12	2 38^{+}0 76	173^{+} 30	415^{+} 260	0 05	5/11
					0 001	1/7
6/7	6	5 18^{+} 1 22	762^{+}425	1040^{+}390	0 001	5/11
					0 001	16/1

Extractabl Insul i hedgehog pancreas at various seasons All figures given are mean $^{+}$ SD

guinea-pig however a reverse pattern for heart glycogen is found (Fig 8 2 and 8 3)

Discussion

Utilization of an immunologic method for the determination of insuli 1 based on the existence of common antigeni ites in the insulin to be determined and that used to produc the antibodies. Insulin in contrast to most proteins has structure whi h shows only small changes between species (Smith 1966) Immunological cross reactivity has been shown between antiserum against bovine insulin and ll mammalian insulins tested with a few exceptions This cross reaction has even been extended to non-mammalian species including some amphibia and fishes (F llner 1969) Among the mammal only some rodents have been found not to share antigenic sites with bovine insuli namely guinea-pig nutria coypu (Myocastor) and capybara (Hydrochoerus) which ll belong to the taxonomi suborder Hystericomorpha of the order Rodentia (Moloney and Coval 1955 Davidson t l 1968 Davidson t al 1969) So à priori it could be expected that insuli from the hedgehog should be possibl to quantitat at least on relative basis with the radio-immuno system with antibovine insuli This did occur If the amount of insuli extractabl from the pancreas i expressed i human insulin equivalents the contents averaged 0 17 IU/g wet weight in the summer and 0 76 IU/g i the winter This is approximat ly of the same order as have been described from some fishes and birds (F llner 1969) but lower than th yield from other mammalian pancreas (Dixit et l 1964 Epstein and Anfinsen 1963 Mirsky et l 1963 F llner and Wilson 1967)

The absenc of any demonstrabl insuli i plasma i the basal state may depend on lower rate of release or higher rat of disappearanc during fasting than i other species studied It may lso be caused by a low immunoreactivity per mole of the hedgehog insuli i thi assay

Fig 8 1 Blood glucose i mg per 100 ml Season efers to the prehibernating (I) hibernating (II) posthibernating (III) and active non-hibernating (IV) phase See further Fig 2 1 in General Methods and Material The solid line efers to hedgehogs the interrupted line to guinea-pigs and the dotted line to homo The number of denote th significance level according to the "t test one means 0 05 > P > 0 01 and two P< 0 01 E efers to the hedgehog (Erinaceus europaeus) C p to the guinea-pig (Cavi porc llus) and H to man (Homo sapiens) 1 indicates the differenc between season I and II 2 between I and III 3 between I and IV 4 between II and III 5 between II and IV and 6 between III and IV Individual values for th human subjects are given i the Appendix tabl

Fig. 8 2 Heart glycogen i % See Fig 8 1 for legend

Fig 8 3 Liver glycogen in % See Fig 8 1 for legend

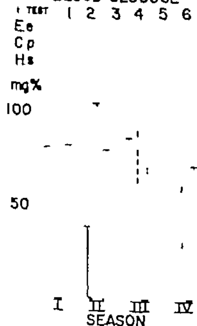


Fig. 8.1

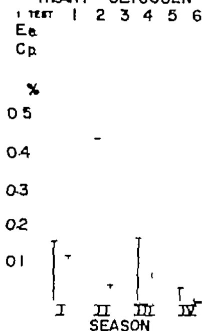


Fig. 8.2

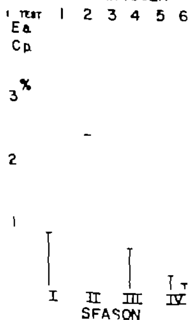


Fig. 8.3

system as compared to the homologous antigen However as no attempts were made to crystallize the extracted insulin these experiments cannot settle this question The r latively low content of insulin in the pancreas may lso be explained by such a fact

The irregular effect of hyperglycemia on insulin release could be an effect of the anesthesia and operative procedure during the test with stress induced elease of adrenalin blocking the release of insuli from the pancreas The observation that blockad f the α-receptors with phentolamine abolished thi influenc suggests that such a mechanism might be in effect during stress

These studies confirm the findings of low blood glucose levels during hibernation while non-hibernators did not show corresponding winter-decrease The similarity between man and the hedgehog probably reflects thei similar diet It has been suggested that the low hibernating blood glucose is the effect of increased secretion of insulin The lack of food intake during hibernation may account for the low blood glucose (Johansson 1972b) no ircul ting insulin could be demonstrated i the basal state i any season by the method used The existance of demonstrabl concentrations of insuli during hyperglycemia indicates that if there had been a marked basal hyperinsulinemia during hibernation this should have been revealed The low concentration of insuli i pancreas during winter has a morphological basis in a decrease of the fraction of β-cells (Zajusz 1957) The decrease i pancreatic insulin preceeded hibernation as low pancreati insuli was ecorded i November Of course the data on the concentration of insulin i the pancreas do not necessarily reflect the rate of insuli elease but they do not support the view of any reduction of stored insulin by hibernation

t

Chapter 9

F t M t bolism I Plasma lipids and lipoproteins

by

Bengt G Johansson and Bengt W Johansson

Despit the obvious nutritional importanc of the yearly cycle of plasma lipids few studies have been performed on the levels of lipids and lipoproteins in hibernators In the present communication we measured the concentrations of total lipids cholesterol triglycerides and non-esterified fatty acids in plasma from hedgehogs taken at different seasons and compared these parameters with those of guinea-pig and man to determine whether the hibernator's plasma lipids showed larger seasonal variations than the two non-hibernating species The results of electrophoreti separations of plasma lipoproteins of the three species will lso be reported

Methods and materials

Total lipid determination was performed as described by Zöllner and Kirch (1962) Analyses of triglycerides and non-esterified fatty acids (NEFA) were carried out as described by Laurell (1966) and Laurell and Tibbling (1967) Determinations of total holesterol wer made using Technicon Autoanalyzer following the method of Boy et l (1960) I a smaller number of animal lipoprot i lectrophoresis was run i agarose gel by a modification of Nobl 's (1968) method (Johansson 1972a) See further chapter 2, General Methods and Materi ls

Results

The results of the lipid analyses of each species ar summarized i Fig 9 1 9 4 It is noted that obvious differences in the total lipid level exist between the guinea-pig on one hand and hedgehog and man on the other The differenc is at least partly due to the low cholesterol content of the plasma of the guinea-pig whereas th content of triglycerides are fairly equal i ll species The guinea-pig however has a considerably higher concentration of NEFA than hedgehog and man These lipid differences are lso reflected i the different plasma lipoprotei patterns seen in Fig 9 5 The i traspecies differences of the lipoprotei patterns were negligibl

TOTAL SERUM FAT

TEST	1	2	3	4	5	6
Ee						
Cp						
Hs						

mg%

1000

500

I II III IV
SEASON

Fig. 9.1

SERUM TRIGLYCERIDES

TEST	1	2	3	4	5	6
Ee						
Cp						
Hs						

mM

1.0

0.5

I II III IV
SEASON

Fig. 9.2

SERUM FREE FATTY ACIDS

TEST	1	2	3	4	5	6
Ee						
Cp						
Hs						

mM

1.25

1.00

0.75

0.50

0.25

I II III IV
SEASON

Fig. 9.3

CHOLESTEROL

TEST	1	2	3	4	5	6
Ee						
Cp						
Hs						

mg%

400

300

200

100

I II III
SEASON

Fig. 9.4

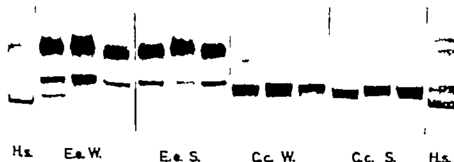


Fig. 9.5

Fig 9 1 Total serum fat I mg % Seasons efer to the prehibernating (I) hibernati g (II) posthibernating (III) and active non-hibernating (IV) phase See further Fig. 2.1 I General Methods and Materi l The solid line efers to hedgehogs the I terrupted line to guinea-pigs and the dotted line to homo The number of denot the igni ficance level according to the "t test one means 0 05 P 0 01 and two P 0 01 E efers to the hedgehog (Erinaceus europaeus) C p to the guinea-pig (Cavi porcellus) and H to man (Homo sapiens) 1 indicates the differenc between season I and II 2 between I and III 3 between I and IV 4 between II and III 5 between II and IV and 6 between III and IV Individual values for the human subjects ar given in the Appendi tabl

Fig 9 2 Serum triglycerides I mM/l See Fig 9 1 for legend

Fig 9 3 Serum non-esterified fatty acids (NEFA = free fatty acids) I mM/l See Fig 9 1 for legend.

Fig. 9 4 Serum total holesterol I mg % See Fig 9 1 for legend.

Fig 9 5 Electrophoreti lipoprotei patterns of hedgehog (E) and guinea-pig (C) during winter (W) and summer (S) Human lipoprot i patterns (H) run as references Electrophoresi performed i agarose gel i barbital buffer pH 8 6

Sinc no haracterization of the lipoproteins was made the fractions could be only tentatively designated as β-lipoprot i pre-β-lipoprot i and α-lipoprotei The predominance of the presumed pre-β-zone of the guinea-pig is striking Hedgehog plasma contains high levels of α-lipoprotei these produc a more complex pattern than that from the guinea-pig The faint components i the plasma lipoprotein pattern of the hedgehog might represent sudanophil components not containing lipids however

The seasonal variations of plasma lipids are summari ed i Fig 9 1 9 4 showing general trend towards lower values during summer or spring for ll three species i human serum the hanges were only significant for cholesterol In the guinea-pig serum however the total lipids were also lowered But the most pronounced variation was found i hedgehog sera The cholesterol values parallelled the hanges of tot l lipids whil the triglyceride values showed somewhat different variation with the lowest values i samples taken i spring The observed variations i NEFA were withi the error of the method The lipoprotei patterns were not ltered for any of the three species Visual inspection of the electrophoretic patterns rul out large hanges i lipoprotein levels. It should be noted that in no cases chylomicrons were see i the lipoprotein lectrophoresi

Discussion

Blood plasma contains several lasses of lipid-transporting lipoprot ins whi h may be separated by preparative ltracentrifugation or lectrophoresis The neutral fat or triglycerides dominate the very low density lipoprot i (VLDL) las It is important to reali e that two types of triglyceride transport in plasma occurs The hylomicrons carry triglycerides from food resynthesized i the i testinal mucosa whereas the triglycerides of endogenous VLDL are formed i the liver by conversion of carbohydrate to fat Both these lipoprotei species undergo continuous lipolysis with deposit of triglycerides especially i adipose tissue The adipose tissue furnishes the organism with easily availabl energy through lipolysi and subsequent transport of fatty acids bound to lbumi NEFA not utili ed by tissues are e-esterified by the liver and agai transported to adipose tissue as VLDL The holesterol transport on the other hand is performed mostly by low density lipoprotein (LDL) or β-lipoprotei and to lesser degree by high density lipoproteins (HDL) or α-lipoprot i

The differences of plasma lipid level between different species should be eflected by differences i lipoprotei distribution Thi is consistent with the demonstrated observations of low β-lipoprot i (and presumably α-lipoprotein) levels i guinea-pig showing low content of cholesterol i plasma The cholesterol ontent is more equal i hedgehog and man The low β-lipoprotei content of hedgehog is obviously compensated for by its higher α-lipoprotei level presumably carrying most of the cholesterol

Seasonal changes of plasma lipid constituents I man have been studied mostly concerning holesterol The results of some studies show that such variations occur I several species with reduced values during summer (Tharp 1963 Thomas et al 1961 Carlsson and Lindstedt 1969) whil other do not find any seasonal variations (Samuel et l 1970 Fleischman et l 1967) Similar variation in triglycerid values have been eported by Carlsson and Lindstedt (1969) Although the small material does not permit conclusions on seasonal changes of plasma lipids I man and guinea-pig except small decrease of cholesterol from winter and to summer the values for the hedgehog learly show such changes which ar most obvious for total lipid and cholesterol Similar seasonal hanges of holesterol levels wer found in hedgehogs (Suomalaine 1954) and I badgers (Johansson 1957) Alexander and Kapeloff (1969) showed high values I three monkeys examined I November and December as compared to July but no change I thr others and decrease I seventh monkey That study was however not well designed t get seasonal information McDonough and Hames (1967) found higher cholesterol values in man I winter than I summer I sedentary individuals however the serum cholesterol I elevated all year long and does not show the clearcut seasonal variation shown by other active occupational groups I diagnosed arterioscleroti patients Samuel et l (1970) found no seasonal variation I serum holesterol

The triglycerides showed less pronounced but statistically significant hanges analogous t the cholesterol As fasting values for all lipids wer obtained the measured triglycerides should probably be included I the very low density lipoprotei of endogenous origi and should therefore be unr lated to the fat I the food but instead lated t an increased onversion of glucose to fatty acids or increased re-esterification I liver This assumption I supported by the esults of lipoprot I electrophoresi which did not show any hylomicron one The rise of NEFA during hibernation shown by Suomalainen and Saarikoski (1967) but t lesser extent I the present work might bear relationship to the increased triglyceride levels by means of an increased re-esterification of the fatty acids presumably I the liver followed by increased output of VLDL to blood A considerabl lipolyti activity has been found I the brown fat during the hypotherm periods (Suomalainen and Saarikoski 1967) Thi activity I probably the cause f the slight increase of the NEFA levels

The constant lipoprot in patterns obtained by agarose gel electrophoresi I different seasons do not rul out small changes I lipoprotei distribution as no quantitative determinations of the fractions were performed The high levels of hedgehog α-lipoprot ins I comparison with man and guinea-pig I striking but before any conclusions concerning the lipoproteins an be drawn haracterization of the lipoproteins I terms f lipoprot in lasses and lipid composition should be performed I addition t an extended investigation of thei yearly patterns. Such study has so far been performed only by Galster and Morrison (1966) using paper lectrophoresi for the separation of lipoproteins from plasma of ground squirrels they got maximal values for total lipid α-lipid β-lipid and hylomicrons I late f ll and minimal values I lat spring

Chapter 10

F t Met b lism II   Fatty acid composition of adipose tissue

by

Björn Åkesson

A low environmental temperature may cause an increase i the unsaturation of animal depot fats (Henriques and Hansen 1901) and lso i human subcutaneous fat (Moriya and Itoh 1969) The occurrenc of this phenomenon i hibernating animals has been tudied (Randinini t l 1962 W lls t al 1965 Spencer et al 1966 Paulsrud and Dryer 1968) but no pattern common to different species was found The seasonal variation i fatty acid composition i brown and white adipose tissue may give information on the metabolic events during the different phases of hibernation and lso eflect the differences i metaboli function between the two tissues (Joel 1965) I thi investigation we have studied the fatty acid omposition of neutral lipids i brown and whit adipose tissue i the hedgehog and i guinea-pig white adipose tissue

Methods and materials

Isolation of neutral lipids The adipose tissue samples (less than 0 5 g) wer homogenized with glass rod in 10 ml chloroform:methanol (2:1) and the mixture was left overnight at room temperatur Then 4 ml 1 % NaH_2PO_4 was added and the lower phase was aspirated after en-)t ifugation The chloroform phase was dried and was applied to silici acid column Neutral lipids wer luted with chloroform

Fatty acid analysi An aliquot of the neutral lipids was transesterified in 2 % H_2SO_4 i *methanol benzene (1 1) at 65 during 4 h The methyl esters wer extracted and purified by thin-*layer chromatography They were analyzed for fatty acid composition by gas-liquid hromatography as described earlier (Åkesson t l 1970) The identity of the fatty acids was established by comparing the etention times with those of authentic standards (obt i ed from the Hormel Institut) The degree of unsaturation of the fatty acids was hecked by thin-layer hromatography on $AgNO_3$ impregnated silic gel Quantitative results from fatty acid analysi of National Heart Institut Fatty Acid Standard F agreed with the tated composition data with a elative error of less than 3 % for major components (10 % of total mixtur) and less than 10 % for minor omponents (< 10 % of total mi ture)

Results

Fatty acid composition in hedgehog adipose tissue The fatty acid composition in whit and brown adipose tissue is shown in Figures 10 1 and 10 2 Only major fatty acids ar shown the amounts of the remaining acids are given in Tables 10 I and 10 II Oleic acid was the most abundant acid followed by palmitic acid whil myristic acid palmitoleic acid stearic acid linoleic acid eicosaenoic acid and docosaenoic acid all ranged between 2 and 9 % of total fatty acids.

The fatty acid composition of brown and white adipose tissue differed in several respects (Table 10 IV) At three seasons or mor the proportion of myristic acid palmitic acid and palmitoleic acid was higher i whit adipose tissue whil stearic acid icosaenoi acid and docosaenoi acid wer more abundant i brown adipose tissue Such dissimilarities may depend on differences in number of metabolic processes (See Discussion)

The following seasonal variations i fatty acid composition wer observed i brown and white adipose tissue (Figures 10 1 and 10 2) The proportion of oelic acid i both tissues decreased during summer whi h was partly compensated for by an increase i linoleic acid i brown adi pose tissue the fatty acids formed by longation i steari acid icosaenoic acid and docosaenoi acid had the same seasonal variation with low values during winter The seasonal changes i hedgehog adipose tissues had littl effect on the degree of unsaturation (Table 10 V) The neutral lipids i brown fat wer mor unsaturated than those i white fat except during summer

Fatty acid composition i guinea-pig adipose tissue I thi tissue ol ic acid dominated over palmitic acid (Figur 10 3 see also Table 10 III) Stearic acid palmitoleic acid linol i acid and linoleni acid wer found i thi order ex ept i samples taken in summer wher the two latter fatty acids attained higher values than stearic acid Thi lee was compensated for by decrease i palmitic acid whereby the degree of unsaturation was considerably increased (Tabl 10 V)

Discussion

The data presented i thi paper will be discussed from the following points of iew a) I which manner are the seasonal variations i fatty acid composition corr lated to the environmental temperature and/or hibernation? b) Do hibernators and non-hibernators differ i these respects?) Finally the differences i fatty acid omposition between brown and white adipose tissues will be discussed

The fatty acid omposition of adipose tissue glycerides or total lipids at different seasons has been studied in three species of bat (Rondini i et l 1962 W lls et al 1965 Paulsrud and Dryer 1968) and i ground squirr l (Spencer et al 1966) I Rinolophus ferrum equinum (Rondinini et l 1962) the amount of polyenoi fatty acids rose t the beginning of hibernation

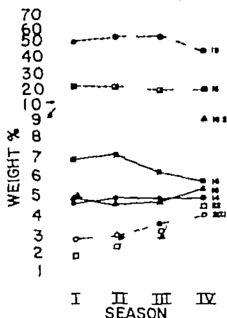


Fig. 10 1

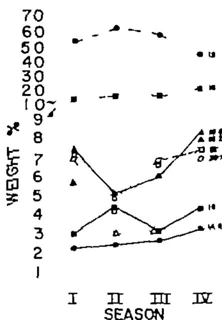


Fig. 10.2

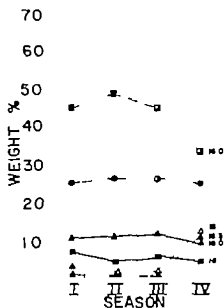


Fig. 10 3

and sank after arousal whereas leic acid decayed to the lowest values during hibernation In brown adipose tissue of *Myoti lucifugus* (Wells et l 1965) hibernation caused decrease in the proportion of olei acid while palmitic acid and palmitoleic acid attained higher values The degree of unsaturation was higher during the non-hibernation period which is l contrast to the findings for *Eptesicus fuscus* (Paulsrud and Dryer 1968) I this animal the percentages of l l acid and linol l acid showed a reciprocity with sharp maximum in linoleic acid during summer This pattern resembles that l the hedgehog (Figures 10 1 and 10 2) I both whit and brown adipose tissues of *Citellus lateralis* the glyceride fatty acid composition varied considerably with no correlation to the hibernation cy le or to the diet (Spencer et l 1966) No common pattern can evidently be seen in the seasonal variations of fatty acid composition for these nimal species. The possibl non-dietary seasonal variations ar difficult to evaluat because of the influenc of diet

Henriques and Hansen (1901) observed that transfer of an animal from warm to cold environment caused an increased unsaturation l the depot fats and suggested that such changes are necessary to keep the fats fluid The same phenomenon was found l the hamster hiber nator (Fawcett and Lyman 1954 Kodama and Pace 1964) but not l the rat non-hibernator (Fawcett and Lyman 1954) Such a differenc between hibernators and non-hibernators was not found in the present study sinc the unsaturation of hedgehog fat did not increase during wi ter (Tabl 10 V) Maybe the proportion of unsaturated fatty acids in the hedgehog l so high that change toward higher unsaturation before hibernation would be unnecessary The degree of unsaturation l the guinea-pig non-hibernator was considerably lower during most seasons (Table 10 V)

Fig 10 1 Seasonal variation l the fatty acid composition of neutral lipids from hedgehog white adipose tissue
The plotted values represent the mean values for the principal fatty acids also given l Tabl 10 l The fatty acids are designated by number of carbon atoms number of doubl bonds (se lso Tabl 10 IV) Abbreviations F f ll; W winter; Sp spring; S summer See further Fig 2 1 l Chapter 2

Fig 10 2 Seasonal variation l the fatty acid composition of neutral lipids from hedgehog brown adipose tissue
The plotted values epresent the mean values for the principal fatty acids also given l Tabl 10 ll See further legend Fig 10 1

Fig 10 3 Seasonal variation l the fatty acid composition of neutral lipids from guinea-pig white adipose tissue
The plotted values represent the mean values for the principal fatty acids lso given l Tabl 10 lll See further legend Fig 10 1

Table 10.I

Seasonal variation in the fatty acid composition of neutral lipids from hedgehog white adipose tissue

Fatty acid	Fatty acid composition				Differences in means[b]					
	Fall	Winter	Spring	Summer	F-W[b]	F-Sp	F-S	W-Sp	W-S	Sp-S
	weight % (mean ± SD)									
14:0	4.4±0	4.7±0.3	4.7±0.8	4.7 [illegible]	-0.3	-0.3	-0.3	0.0	0.0	0.0
16:0	21.5±2.5	20.7±1.2	18.7 [illegible]	18.9±3.0	0.8	2.8***	2.6	2.0**	1.8	-0.2
16:1	6.8±1.3	7.0±0.6	6.1±0.9	5.4±1.4	-0.2	0.7	1.2	0.9*	1.6*	0.8
18:0	4.7 [illegible] 4	4.5±0.6	4±0.4	5.3±1.5	0.2	0.1	-0.6	-0.1	-0.8	-0.7
18:1	52.4±2.8	53.8±2.0	54.9±2.0	45.5±7.4	-1.4	2.5*	6.9***	1	8.3***	9.4***
18:2	4.9±2.6	2.7±0.9	2.8 [illegible] 1	8.9±3.4	2.2*	2.1	-4.0*	-0.1	-6.2***	-6.1***
18:3+20:0	0.9±0.6	0.6±0.1	0.6±0.2	1.6±0.5	0.3	0.3	-0.7***	0.0	1.0***	1.0***
20:1	2.8±0.9	2.7±0.5	2.3±0.6	3.8 [illegible]	-0.2	1.0***	-1.3***	-0.8***	1.1***	-0.3
20:2		0.5±0.3	0.4±0.2	0.3±0.1				0.1	0.3	0.2
22:0		0 [illegible]		0.3±0.3					-0.4	
22:1	[illegible]±1.8	2.1±0.7	2.0±0.8	4.3±2.0	-0.3	[illegible]***	2.5***	-0.9*	-2.3***	[illegible]
others	0.8±0.5	0.9±0.3	1.0±0.3	0.7±0.2	-0.1	[illegible]	0.1	-0.1	0.2*	0.3***

Data are means from 10-12 animals.

[b] Abbreviations: F, Fall; W, Winter; Sp, Spring; S, Summer

* 0.01 < P < 0.05 (t-test)

** 0.001 < P < 0.01

*** P < 0.001

Table 10.II

Seasonal variation in the fatty acid composition of neutral lipids from hedgehog brown adipose tissue

Fatty acid	Fatty acid composition				Differences in means					
	Fall	Winter	Spring	Summer	F-W[a]	F-Sp	F-S	W-Sp	W-S	Sp-S
	weight % (mean ± SD)									
14:0	2 [illegible]±0.5	2.3±0	2.5±0.6	3.1±1.1	[illegible]	-0.4	[illegible]*	[illegible]	-0.8*	[illegible]
16:0	13.3±1.8	14.3±0	14.3±1	18.0±3.6	1.2*	0	-4.7***	0.2	-3.5***	-3.7***
16:1	2.9±0.6	3±0	0±0.9	3±1	[illegible]***	-0.1	[illegible]***	[illegible]***	0.1	[illegible]***
18:0	7 1±	4.8±0	9±1.1	7.9±[illegible]	3***	[illegible]	-0.8	1***	3.1***	2.0***
18:1	33.8±8.6	69.9±3	65.2±6.0	42.4±6.3	-4.5***		9***	4.7*	17.3***	12.8***
18:2	3.7 [illegible]3	2.9±	3.0±2.3	7±3	2.5***	2.7***	0	-0.1	-4.8***	-4.7***
18:3+20:0	1.3±0	1.0±0	1±0.8	1.1±0	0.2	0.	0.	-0.1	-0.1	0.0
20:1	6.9±	4.6 [illegible] 8	4±1.7	6.6 [illegible] 7	2.3***	0.3	0.3	[illegible]***	[illegible]*	-0.2
20:2	0.4±0.3	0.5±0	7±0.4	0.2±0	[illegible]	-0.3	0	-0.2	0.3*	-0.3*
22:0		0.4±0.4	0.3±0	.3±0				0.	0.1	0.0
22:1	6.7±3.6	3±	6.6±4.4	7.4±3.9	2.5	1	-0.7	-2.	-3.7*	-0
Others	0 [illegible]±0	1.1±0.	3±0.7	4±0	-0.4***	-0.5*	-0.7*	-0.1	-0.3	-0.2

Abbreviations see Table 10.I.

Table 10.III.

Seasonal variation in the fatty acid composition of neutral lipids from guinea-pig white adipose tissue.

Fatty acid	Fatty acid composition				Differences in means					
	Fall	Winter	Spring	Summer	F-W	F-Sp	F-S	W-Sp	W-S	Sp-S
	weight % (mean ± SD)									
14:0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
16:0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
16:1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
18:0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
18:1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
18:2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
18:3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
20	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
20:1	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]			[illegible]
Others	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Abbreviations see Table 10.I.

Brown and white adipose tissue have been compared with respect to the glyceride fatty acid composition i rat (Chalvardjian 1964) mouse (Spencer and Dempster 1962) and golden-mantled squirrel (Spencer et al 1966) Through ll studies including the present one (Tabl 10 IV) the proportion of steari acid is higher in brown adipose tissue while palmitoleic acid is higher i white adipose tissue The larger amount of steari acid i brown fat was also noted in hamster (Smalley et l 1970) and rabbit (Toki and Takatori 1969) These discrepancies between the two tissues may eflect differences i the enzymatic equipment for the synthesis oxidation and interconversion of fatty acids and also i the spectrum of fatty acids extracted from and secreted to blood plasma Some evidenc on such selectivity is availabl i the literature More polar free fatty acids or more readily released from and esterified i rat adipose tissue (Hollenberg and Angel 1963) Kodama and Pace (1964) suggested that the increased unsaturation i hamster fat upon cold exposure was achieved by an increased fat mobilization that was selective with respect to indi idual fatty acids Changes i fatty acid composition during hibernation when the diet does not influence may depend on diffe- rent oxidation rates for different fatty acids (Paulsrud and Dryer 1968) These authors lso found that lowering of the temperature produced preferenti l oxidation of palmitic acid over oleic acid in bat brown adipose tissue. Sinc brown adipose tissue had the highest amounts of the longer fatty acids i steari acid eicosaenoi acid and docosaenoi acid (Tabl 10 IV)

this tissue may contai more of the fatty acid elongating enzyme(s) This may in turn be related to its higher content of mitochondri (Joel 1965) If the elongation by ac tyl-CoA as in the liver (Harlan and Wakil 1963) takes place in thi organell The lative roles of ll these processes for the fatty acid changes reported in thi paper can of course not be assessed until more experimental evidence becomes availabl

Tabl 10,IV

Comparison between hedgehog brown and whit adipose tissue with respect to the fatty acid composition in neutral lipids

Fatty acid	Differenc in means[a]			
	Fall	Winter	Spring	Summer
Myristi acid 14 0[b]	2 3[xx]	2 4[xx]	2 2[xx]	1 6[xx]
Palmiti acid 16:0	8 2[xx]	6 2[xx]	4 4[xx]	0 9
Palmitoleic acid 16:1	3 9[xx]	2 7[xx]	3 1[xx]	1 4[x]
Stearl acid 18:0	2 4[xx]	−0 3	1 3[xx]	2 6[xx]
Oleic acid 18:1	−0 9	−6 1[xx]	−0 3	3 1
Linol i acid 18 2	−0 8	−0 2	−0 2	1 2
Eicosenoi acid 20:1	−4 4[xx]	1 9[xx]	2 9[xx]	2 8[xx]
Docosenoi acid 22:1	−4 9[xx]	2 1[xx]	3 6[x]	−3 1[x]

[a]Values for whit adipose tissue minus values for brown adipose tissue

The statistical significance was examined by t-test

0 01 < P < 0 05

[xx]0 001 < P < 0 01

[b]The abbreviation denotes no of carbon atoms: no of doubl bonds

Tabl 10,V

Mean doubl bond content per mol fatty acid during different seasons

The data wer lculated from Tables 10 I 10 III

Tissue	Season			
	Fall	Winter	Spring	Summer
Hedgehog whit adipose tissue	0 76	0 74	0 76	0 82
Hedgehog brown adipose tissue	0 86	0 83	0 82	0 80
Guinea-pig whit adipose tissue	0 42	0 36	0 39	0 86

Chapter 11

Dehydrog n s (LDH MDH G-6 PDH d a GPDH) in the H art Liv Whit nd Brown F t

by

Sven-Oll R Olsson

Contents

I INTRODUCTION

The dehydrogenases studied here have more or less key positions in the carbohydrate metabolism as I illustrated I figur 11 1

Lacti dehydrogenase (LDH) (EC 1 1 27) catalyzes the final step I anaerobi catabolism of glucose

Malic dehydrogenase (MDH) (EC 1 1 1 37) participates in the Krebs'cycle which I of importance for aerobic catabolism MDH lso functions I gluconeogenesis.

Glucose-6-phosphate dehydrogenase (G-6-PDH) (EC 1 1 1 49) catalyzes the first step in the pentose-shunt which is important for the production of $NADPH_2$ and ribose

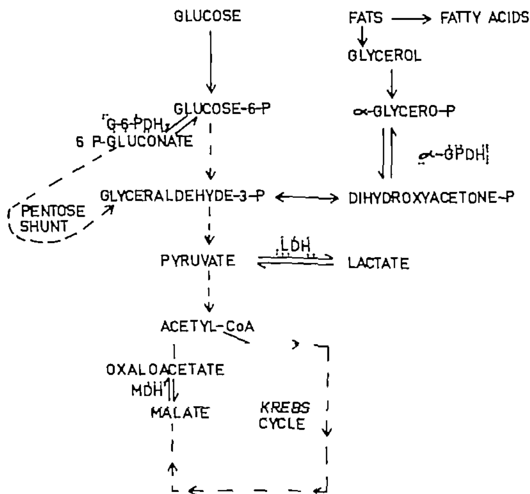
α-Glycerophosphate dehydrogenase (α-GPDH) (EC 1 1 1 8) links glycerol catabolism with glycolysis

These four enzymes were studied in hedgehogs both hibernating and non-hibernating and I guinea-pigs whi h wer killed during four periods of the year (Chapter 2)

The multipl molecular forms (isoenzymes) of LDH and MDH gained particular interest

One of the most obvious differences between hibernators and non-hibernators I the cold-resistance of tissues from hibernators. These tissues function at much lower temperatures than do those of non-hibernators. The organ which has been tudied most extensively I this respect is heart (Johansson 1967 1969a) Makrash et al (1960) reported lower Q_{10}-values for heart rat I isolated preparations from hibernating hamsters than from non-hibernating hamsters and rats but these results are not supported by Mi hael and Menaker (1963) or Senturi et al (1970) who worked with bats and ground squirrel respectively The Q_{10} values for oxygen onsumption of ventri les have lso been found to vary The value for rat ventri les was found to be higher than that for non-hibernating hamster ventri les which I turn is higher than the values from hibernating hamsters and bats (South 1958) Essentially the same results have been found from ats and hibernating and non-hibernating hamsters for oxygen consumption of isolated mitochondria from ventri les (South 1960 and b) and for esterification of inorgani phosphate by mitochondria of the ventricles (South 1960 and b) These results show that there is enzymatic difference I temperature resistance between the non-hibernator (rat) and the hibernator (hamster) and that in hamsters there evidently I an enzymatic adaptation to hibernation

During recent years many enzymes have been found t be composed of different protei structures (isoenzymes) whi h however catalyz the same reaction (Wilkinson 1965 and Latner and Skillen 1968) The enzyme which I best known I this espect I Lacti dehydrogenase LDH I omposed of five isoenzymes and every isoenzyme consists of tetramer Two types of subunits H and M are randomly distributed I the five tetramers (isoenzymes) LDH-1 onsists of four H-subunits and LDH-5 consists of four M-subunits The other three isoenzymes consist of 3H and 1M (LDH-2) 2H and 2M (LDH-3) and 1H and 3M (LDH-4) These five isoenzymes ar distributed according to the binomi l distribution I each kind of cell (Markert 1963 Kaplan and Cahn 1962



GLUCOSE
FATS
FATTY ACIDS
GLYCEROL
GLUCOSE-6-P
α-GLYCERO-P
G-6-PDH
6 P-GLUCONATE
α-GPDH
GLYCERALDEHYDE-3-P
DIHYDROXYACETONE-P
PENTOSE
SHUNT
LDH
PYRUVATE
LACTATE
ACETYL-CoA
OXALOACETATE
MDH
MALATE
KREBS
CYCLE

Fig. 11.1

and Boyer et _l_ 1963)

If the relative activities of the two subunits are H and M (H + M = 1) the activities of the isoenzymes are the expansion of $(H + M)^4$ Palmer and Karlsson (1969) have shown that liver c lls free from connective tissue have an LDH-distribution which fits the binomial di tribution better than LDH from whole liver or from onnective tissue Thei conclusion is that each cell line has a specifi ratio of H/M and a binomi l distribution of the activities of H and M. The synthesis of two subunits i controlled by different genes whose changes i activity result i changes i the relative activities of the isoenzymes These hanges have been demonstrated during development of mammals (Karlsson and Palmer 1971)

The distribution of LDH-isoenzymes i different organs has been corr lated to the metabolic functions of that organ Dawson et _l_ (1964) pointed out that organs with anaerobic metabolism have a high proportion of LDH-M (_g_ keletal muscl and whit fat) and that organs with aerobic metabolism have hig proportion of LDH-H (_g_ heart and brain) Wilson _t al_ (1963) investigated the LDH-isoenzymes from the pectoralis muscl from more than 30 species of birds and found that skillful flyers such as swifts had higher proportion of LDH-H then domesti fowl and game birds. It is generally accepted that muscles which work mor or less continuously (I__ the myocardium or pectoralis muscle) have higher activity of LDH-H than those muscles whi h work intermittently such as extremity muscles (Dawson et _al_ 1964 Kaplan 1964 Lindsay 1963) The later group of muscles with high proportion of LDH-M will produce lactat and increase the oxygen debt at increased work. The reason for this is that LDH-H is inhibited mor by increasing concentrations of pyruvat than LDH-M (Cahn et _l_ 1962) Salthe (1965) found that there is differenc between the distribution of LDH i terrestrial aerobic-living and i aquati anaerobic living amphibians I vitro the synthesi of LDH-M is inhibited (Goodfriend and Kaplan 1963 Dawson et _al_ 1964) and synthesis of LDH-H activated by increasing the PO_2 (Cahn 1963 1964) Lindy and Rajasalmi (1966) incubated eggs i hypoxi and hyperoxi environments The hypoxic eggs had higher activity of LDH-M than the hyperoxic eggs. It is possibl that oxygen content i the tissues may be regulating factor for the synthesi of LDH-H and LDH-M and that tissues with aerobic metabolism have high activity of LDH-H Burlington and Wiebers (1966) showed that the capacity of the myocardium for anaerobic glycolysi increased during hibernation and that thi increased capacity is i connection with the hypoxi tolerance of the hibernators (Biörck et _l_ 1956a Kayser and Malan 1963 Andjus _t l_ 1964) Burlington and Sampson (1968) found that LDH-M increased i the liver brai and heart of the hibernating ground squirrel (_Citellus tridecemlineatus_)

Fig 11 1 A schematic drawing of the principal metabolic pathways i which LDH MDH G-6-PDH and α-GPDH are involved.

Malate dehydrogenase onsists of two isoenzymes cytoplasmic (C MDH) and mitochondrial (M-MDH) Kaplan (1963) proposed the following metabolic rol for these isoenzymes: C MDH reduces oxaloacetate and M-MDH oxidizes malate A similar role for the mitochondrial and cytoplasmatic α-GPDH has been proposed by Bücher and Klingenberg (1958) and Sacktor (1959)

I this study enzymatic adaptations to hibernation and temperature resistanc has been investigated in the following ways:

1 The activities of LDH MDH G-6-PDH and α-GPDH from the heart, liver whit and brown fat of the hedgehog were compared to the same organs of the guinea-pig

2 The seasonal changes of these enzymes in the above mentioned organs wer examined

3 The temperature dependence of the acti ity of LDH from the heart of the hedgehog was determined

4 The annual hanges and differences f the isoenzymes of LDH and MDH from the heart of the hedgehog and gui ea-pig were also studied

General methods

The tissue samples from heart liver white and brown fat whi h were taken from hedgehogs and guinea-pigs (Chapter 2) were homogeni ed with knif homogenizer (Sorvall) in distilled water to produc 10% (by weight) homogenates These were entrifuged at 2000 x G for 30 minutes at 4 C and the supernatants were saved for determination of enzyme activities and isoenzyme distribution

Determination of the concentrations of solubl proteins Solubl proteins were determined in order to get a rough estimation of thei concentration changes with a technically simpl and qui k method i order to get a point of eferenc for the enzyme changes (Palmer and Karlsson 1969 Karlsson and P lmer 1971) As thi method described by Layne (1957) i unspecific only large changes can be observed The standard deviation i per cent of the concentration of soluble prot ins i the different organs i 30% $^{+}$ 10% Thi i an expression of the individual variations and the inaccuracy of the method A control experiment with 12 pieces of liver from one *guinea-pig gave standard deviation of 23% which i less than the value (30%) for all 48 livers of the guinea-pigs* Thi means that the inaccuracy of the method of determination contributes greatly the standard deviations

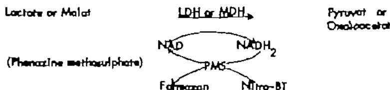
Determination of the enzyme acti ities The activities of the dehydrogenases: lacti dehydrogenase (LDH) (EC 1 1 1 27) malt dehydrogenase (MDH) (EC 1 1 1 37) glucose-6-phosphate d hydrogenase (G-6-PDH) (EC 1 1 1 49) and α-glycerophosphat dehydrogenase (α-GPDH) (EC 1 1 1 8) wer determined spectrophotometrically (Unicam SP 800-A) The activity was measured from the initi l velocity of eduction of the reactions NAD → $NADH_2$ and NADP→$NADPH_2$ at 340 mμ using distilled water as referenc The reactions were performed

at 30 0° C ± 0 05° C

The eactions were started by adding the supernatant of the homogenate to the rest of the eaction mi ture It took about ten seconds to add the supernatant and shake the cuvettes befor the automatic spectrophotometric measurement started If the activity was too high i e the change of extinction during the first minute was non-linear the supernatant was diluted until a linear curve was obtained The changes for extinction were continuously egistered for three to five minutes

The activities ar expressed i activity/wet-weight the change in extinction during one minute per 10 µl 10% supernatant

Determination of the isoenzyme distribution LDH and MDH from the heart Horizontal starch gel electrophoresis was used (Fine t al 1963) as modified by Karlsson and Carlsson (1968) The lectrophoretic separation were run for 18 hours at 4° C using onstant current of 15mA per gel Four degrees Celsius was selected to avoid heat inactivation of the enzymes The development of the isoenzyme bands is based on the reactions:



These reactions were stopped after 20 minutes by incubation at 20° C with 3% aqueous formaldehyde

The amount of insoluble Formazan i the bands was spectrophotometri lly measured with Chromoscan densitometer (Joyc Loebl and Co Ltd.) and taken as measure of the activity of the individual isoenzymes. Latner and Skillen (1968) and Palmer and Karlsson (1969) noted that the absolute acti ity of the LDH-isoenzyme cannot be onsidered directly proportional to the amount of dye i ll experiments performed Considering this the acti ity of the individual isoenzymes are expressed as relative percentage f the total area of the densitogram

II PROTEIN

Method

The determination of the soluble prot in concentration (mg/10 µl) in the homogenat supernatants was performed by the spectrophotometri method of Layne (1957) using a Beckman DB spectrophotometer

Results

Heart See Fig. 11 2

Liver See Fig 11 3

White fat See Fig 11 4

Brown fat See Fig 11.5

Comparison between the organs The highest oncentration of solubl proteins is found in liver and brown fat from the hedgehog and the liver of the guinea-pig

Discussion

The hanges i the concentration of the solubl proteins from these four organs cannot definit ly be correlated with the weight of the organ (Chapter 3) or the concentration of glycogen i the heart and the liver (Chapter 8) Whitten and Kl l (1968a) found decrease i protei synthesi methionine i hepati tissue of hibernating ground squirr l (Citellus tridecemlineatus) at 37° C compared to the non-hibernatory stat whereas no change was observed at 6° C Whitten (1971) has lso observed that decreased protei synthetic capacity i hibernating animals was primarily due to ribosome disaggregation rather than change i the activity of ll sap enzymes

The high concentration of prot ins i the brown fat of the hedgehog during hibernation and post hibernation is i agreement with the results of Paleus and Johansson (1968) who found higher lipidfree dry weight during hibernation i hedgehogs and F lst and Quay (1969) who found an increased concentration of proteins and a decreased concentration of lipids i coldacclimated hibernating and aroused hamsters (Mesocricetus) This i an expression of the higher

Fig 11 2 Concentration of solubl proteins i mg/10 µl The season efer to the prehibernating (I) hibernating (II) posthibernating (III) and non-hibernating (IV) phase See further Fig 2 1 The ircles represent the mean and the straigt lines epresent the standard deviations solid line hedgehog interrupted line guinea-pig

Fig 11 3 For legends see Fig 11 2

Fig 11 4 For legends se Fig 11 2

Fig 11 5 For legends see Fig 11 2

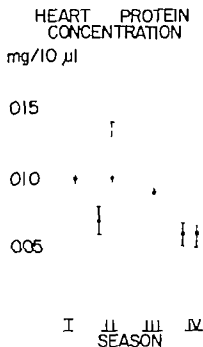


Fig. 11.2

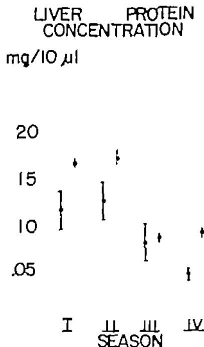


Fig. 11.3

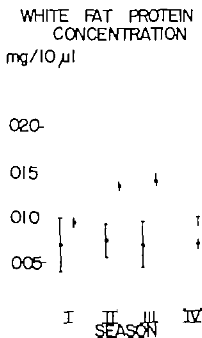


Fig 11.4

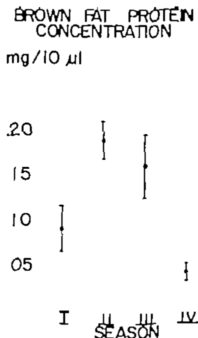


Fig 11.5

metabolic activity of brown fat during hibernation and post hibernation (Draskoczy and Lyman 1965 Edwards and Munday 1969a and Chapter 15)

It appears to be more relevant to express the activities of enzymes in activity/wet-weight of the organs rather than in activity/mg protei inc the effi iency of the enzymes for the whol organ depend primarily on the total activity of the enzymes of the organ If there ar great hanges in the conc ntration of soluble proteins (which is actually the ase i liver heart and white fat of guinea-pig and i brown fat of hedgehog) the activity/mg protei of enzymes does not reflect the hanges i importanc of enzymes for the organs but reflects the changes in concentrations of soluble proteins Another method of expressing activity of enzymes is activity/dry weight and according to Kristofferson et al (1965) the per cent water content of heart liver white and brown fat do not vary more than two per cent of the wet-weight Paleus and Johansson (1968) found that water content i per cent of wet-weight of brown fat f hedgehogs varies maximally 14% during the year The conclusions of these results ar that variations in water content in these organs do not have significant influence on the values of activity/wet-weight

Methods

Determination of the activity of the enzym The general principles of determination of dehydrogenase activity have already been discussed The eaction mixture for the spectrophotometric measurement was as follows

10 µl homogenat supernatant

2.5 ml 0 1M Tris-buffer pH 8 9

0 3 ml 3M Li-lactat (B D H or Sigma)

0 2 ml NAD 10 mg/ml (Sigma)

The reaction was started by adding the supernatant of the homogenat to the rest of the reaction-mixture

Determination of the relative activity of the isoenzymes The technique of electrophoresis was modified after Fine et al (1963) The starch gel (Connaught Med Res Lab Toronto) used here was 13% (weight %) i 0 01 M phosphate-citrat buffer pH 7 0 25 µl homogenat supernatant of tissue was applied i every slit Ther were four slits per gel The lectrophoresi tests wer run for 18 hours i a 4^{0} C environment The urrent was constant at 15 mA per gel (220x90x7 mm) After the lectrophoresi each gel was sliced into two halves one upper and on lower part The lower half was incubated with stai ing solution i total darkness at 20^{0} C for 20 minutes The st ining solution had the following composition:

200 ml 0 2M T is-buffer pH 8 6

6 ml 2M Li lactate (B D H or Sigma)

2 4 ml 30 mg/ml NAD (Sigma)

4 ml 10 mg/ml Nitro-BT (Nitro-Blue-Tetrazolium Sigma)

1 ml 5 mg/ml PMS (Phenazine Methosulphate Sigma)

Determination of the activity of LDH-H i order to determine the activity of LDH-H it is necessary to suppose that the LDH-isoenzymes have binomi l distribution according to the formula:

$H^4 + 4H^3M + 6H^2M^2 + 4\,HM^3 + M^4$ total-LDH-activity

With thi formul and the experimentally determined distribution f acti ity of isoenzymes LDH-H and LDH-M have been calculated as per cent activity of total activity according to P lmer and Karlsson (1969) and Karlsson and Palmer (1971) I order to prove that the distribution really is binomi l tests hav been made through di ect comparison of the observed distribution to the calculated binomi l di tribution from the values of LDH-H and LDH-M

Determination of the energy of activation of LDH I order to determine the energy of activation (µ cal/M) the enzyme-activity was measured according to the above described method at different temperatures from 0^{0} C to 70^{0} C The energy of activation was calculated between

0° C to 50° C with the Arrhenius equation (Precht _t 1_ 1955)

$$\mu = \frac{4.574 \times \log K_2/K_1}{1/T_1 - 1/T_2}$$

K_1 and K_2 are the activities at the temperatures T_1 and T_2 ($^{\circ}$K)

Results

Heart See Fig 11 6

I hedgehog the LDH-activity is higher during winter and summer than I the guinea-pig

Liver Se Fig 11 7

I hedgehog liver th LDH-activity is about five times greater than i guinea-pig liver

White fat See Fig 11 8

During the winter LDH-activity of hedgehog white fat is greater than that in guinea-pigs

Brown fat See Fig 11 9

The activities of the LDH-isoenzymes from the heart of the hedgehog and the guinea-pig. The distribution of LDH-isoenzymes from hedgehog heart (Fig. 11 10) but not that from guinea-pig heart (Fig 11 11) f llows the binomial distribution This implies that the figures for guinea-pig LDH-H acti ity (1 % of total) are very uncertain as these are calc lated on the assumption that the distribution I binomi I (Fig 11 12)

The LDH-isoenzymes hange during the seasons (Fig 11 12) Between autumn and winter (I-II) the activity of LDH-H decreases significantly and between spring and summer (III IV) this acti ity increases almost significantly (0 05 P 0 10) (Fig 11 12)

The energy of activation of LDH from the heart of the hedgehog (Fig 11 13) The energy of activation of LDH from hedgehog heart has ignifi antly (P = 0 05) higher values during autumn and winter (I-II) than during spring (III) The increase of energy of activation between

---

Fig 11 6 LDH-activity i units described i the text The solid line refers to hedgehogs and the interrupted line to guinea-pigs The number of denote the significanc level according to the "t test one means 0 05 > P > 0 01 and two P < 0 01 E. refers to the hedgehog (Erinaceus europaeus) and C p to the guinea-pig (Cavia porc llus) 1 indicates the differenc between seasons I and II 2 between I and III 3 between I and IV 4 between II and III 5 between II and IV and 6 between III and IV See further legends Fig 11 2

Fig. 11 7 LDH-activity i units described i the text For legends see Fig 11 6

Fig 11 8 LDH-activity i units described i the text For legends see Fig 11 6

Fig 11 9 LDH-activity in units described i the text For legends see Fig. 11 6

HEART LDH

"t" TEST 1 2 3 4 5 6

Ee

Cp

act/wet wt

0.2

01

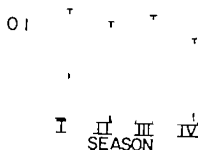


Fig. 11.6

LIVER LDH

"t" TEST 1 2 3 4 5 6

Ee

Cp.

act/wet wt

05

04

03

02

01

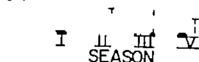


Fig 11.7

WHITE FAT LDH

"t" TEST 1 2 3 4 5 6

Ee

Cp

act/wet wt

0 02

001

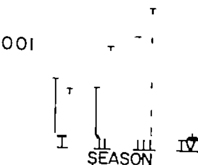


Fig 11.8

BROWN FAT LDH

"t" TEST 1 2 3 4 5 6

Ee

act/wet wt

0 1

0 05



Fig. 11.9

spri g and summer (III IV) is uncertain as the coefficient of orrelation (for linear regression) for summer material is only -0 94

Discussion

The activity of LDH from hedgehog heart is higher than that of guinea-pig and the former activity increases during hibernation This may imply that LDH has a great importance during hibernation

Burlington and Wiebers (1966) have shown that myocardium of hibernators has an increased capacity of anaerobic metabolism as compared to non-hibernators Hiestrand et al (1950) have shown that hibernators have an increased resistanc to hypoxia i comparison with non-hibernators Kayser and Malan (1963) and Andjus et l (1964) have shown that hibernators have an increased apacity for cerebral anaerobic glycosis as compared to non-hibernators Furthermore Covino and Hannon (1959) and Hannon et al (1961) observed that myocardial tissue hows increased capacity for anaerobic metabolism duri g hibernation Zimny (1956) found that ATP levels decrease i the myocardium during hibernation and that anaerobi glycolysi i inversely dependent on ATP concentration (Kapla 1951) In this work Johansson and Senturi (Chapter 6) have described Higher PCO_2 blood standard bicarbonat and lower pH i hibernating hedgehogs in comparison with the non-hibernating hedgehogs Popovic (1964) found decreased arterial oxygen pressure in hibernating ground squirrel i comparison with normothermic animals. If hibernators with thei highly efficient anaerobic metabolism are hypoxic during hibernation the concentration of lactat i the blood should increase Hanson and Johansson (1961) and Twent and Twent (1968) did not find any hanges i lactate concentration in any orga Agid and Ambid (1969) confirmed that the levels of both plasma glucose and lactate ar extremely low i the hypothermic garden dormouse Eliomys quercinus. Duri g arousal the levels of plasma FFA and lactat increase and the amount of oxygen in ar teri l blood decreases They suppose that the energy for the first stage of arousal originates from anaerobi glycolysi as suggested by the increase in lactat

Fig 11 10 Percental activity of the LDH-isoenzymes of hedgehog (LDH-1 ◇ LDH-2 ▲ LDH-3 ⬭ LDH-4 o LDH-5 □) Binomi l distribution of the LDH-isoenzymes based on the calculated LDH-H activity (LDH-1 ◆ LDH-2 ▲ LDH-3 ⬬ LDH-4 LDH-5 ■) For further legends see Fig 11 6

Fig 11 11 Percental activity of the LDH-isoenzymes of guinea-pig For further legends see Fig 11 10

Fig 11 12 Percental activity of tot l LDH-H ⬤ hedgehog ○ guinea-pig

Fig 11 13 Energy of activation for LDH from heart of hedgehog P = 0 05 in "t" test between ither autumn (I) or winter (II) and spring (III)



Fig 11 10

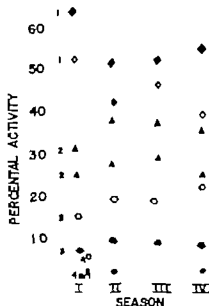


Fig. 11 11

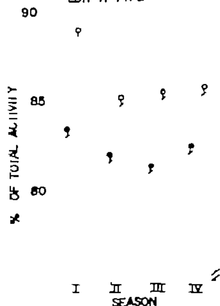


Fig. 11 12

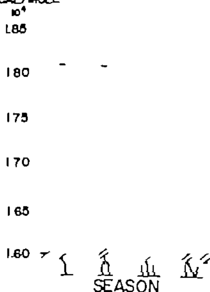


Fig 11 13

On the other hand Burlington and Klai (1967) observed that during hypoxia the concentration of lactic acid increases i serum of the ground squirrel (Citellus tridecemlineatus) and simultaneously gluconeogenesis from pyruvat glycerol α-ketoglutarat ℒ-glutamate ℒ-aspartate and oxaloacetat in kidney cortex slices increases The rat reacted towards hypoxia by decreasing gluconeogenesis and by keeping the concentration of lacti acid constant This shows that the hibernator as distinguished from the rat ompensates for hypoxi with anaerobi metabolism and gluconeogenesis. I kidney ortex sli es Burlington (1966) has shown that the capacity for gluconeogenesis increases duri g cold exposure both in rats and in hamsters

Zimny and Moreland (1968) correlated the low activity of succini dehydrogenase i heart of the ground squirrel Citellus tridecemlineatu with the decrease in the rat of aerobi metabolism during hibernation Tashima et l (1970) found that glycolytic intermediates are specifi cally blocked from entering the Krebs cycl during hibernation They also stressed that the hibernator resembles thiamine-deficient animal with pyruvate dehydrogenase insuffi iency (Weiner and Steyn-Parvé 1959) Chaffee et al (1961) Denyes and Carter (1961) Denyes and Hassett (1960) South (1960 and b) and Baumber and Denyes (1963) have ll observed an increased capacity of oxidativ metabolism i cold acclimated and hibernating hamsters It is possibl that ther is species difference between hamsters (Mesocricetus) and ground squirrels (Citellus) i thi respect

The changes of activities of LDH-H and LDH-M i myocardium (Fig 11 12) suggest that ther is displacement towards anaerobi conditions during hibernation This idea is supported by the work of Burlington and Sampson (1968) who found that the acti ity of LDH-M in myocardium increased during hibernation

The rat non-hibernator hows decreased activity of LDH-M i myocardium during cold stress (Blatt t l 1965) whi h indicates that aerobi metabolism has increased As noted above the rat i not as capabl of anaerobi metabolism as the hibernator (Burlington and Klain 1967) Another non-hibernator man increases activity of LDH-M i serum during cold adaptation (Mager t l 1968) Simmonds et l (1971) did not find any hanges of LDH-isoenzymes in serum of hibernating and non-hibernating ground squirrel Citellus mexicanus It i very difficult to corr late these results from different tissues myocardium and serum Thi increase of LDH-M activity in myocardium during hibernation cannot explain the changes of the heat of activation of LDH (Fig 11 13) Organs with high activity of LDH-M for instanc liver have a lower energy of activation than organs with high activity of LDH-H for instance heart from hedgehog the bat (Nyctalus noctul) and guinea-pig (Olsson 1971) which Plagemann t al (1961) found i rabbits LDH from the pectoralis muscl of Nyctalus noctul has an even higher value for heat of activation than LDH from the heart of N noctula This i due to higher activity of LDH-H i pectorali muscl than i heart (Olsson 1971) The discrepancy between the results from hanges in energy of activation of LDH and from hanges of the LDH-H/LDH-M

ratio can of course depend ither on methodological errors or thermal changes of the sterical conformation of LDH-2 3 or -4 Johansson (1969) also showed these changes of the heat of activation and Burlington and Sampson (1968) also showed changes I the LDH-H/LDH-M ratio The hanges I heat of activation of LDH from hedgehog heart or rather small (about 7%) (Fig 11 13) and should have little importanc during hibernation but can reflect greater changes I other enzyme systems The heats of activation for heart rate of the ground squirrels Citellus tridecemlineatus and C mexicanus or 2 2 x 10^4 and 2 0 10^4 c l/M respectively (Senturi et al 1970) The heat of activation for heart at I hedgehogs is about the same: 2 3 x 10^4 cal/M calculated from the values of Kristoffersson and Soivi (1964): 8 beats/ml body temp: 5 C and Biörck and Johansson (1955): 181 beats/ml body temp: 32 5 C It is evident from these values and from the value for the heat of activation for the activity of LDH from hedgehog heart (1 8 1 7 10^4 cal/M) that LDH cannot be the limiting step I the temperature dependence The heat of activation of LDH from guinea-pig heart is even lower (1 6 x 10^4 cal/M) (Olsson 1971)

Li et al (1969) found an increased Q_{10}-value for oxygen consumption of liver of hibernating ompared to non-hibernating ground squirrels (C tridecemlineatus) but South (1958 1960 and b) found decreased Q_{10}-value from the hibernating compared to the non-hibernating hamster (Mesocricetus) Whitten and Klain (1968a) did not observe any differences I the Q_{10} of arginase activity or protei synthesis from methionine I liver of hibernating or normothermal Citellus Further studies on the temperature dependance of essential enzyme systems or needed and will I the future give valuabl information about the evolution of homeothermism and hiber nation

The changes of the LDH activity from the heart and the liver of the hedgehog or lmost the same with maximal values during hibernation These results are supported by the work of Burlington and Klai (1967) and Burlington and Sampson (1968) It might be part of compensatory mechanism for the low body temperature during the hibernation

I plasma and erythrocytes of hibernating ground squirrel (Citellus mexicanus) Simmonds et l (1971) found maximal LDH-activity but I serum of hedgehogs guinea-pigs and man there were no ignificant hanges I LDH-activity betwee the seasons (Fig. 11 14) There were lso no significant seasonal hanges I GOT- (Fig 11 15) or GPT (Fig. 11 16) activity from serum of hedgehogs guinea-pigs or man However the activity of GPT from summer guinea-pigs is ignificantly lower than from the other seasons (Fig. 11 16)

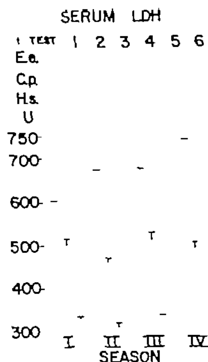


Fig. 11 14

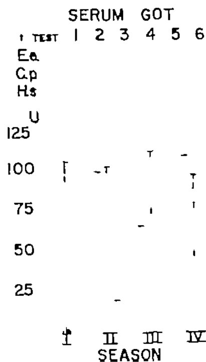


Fig. 11.15

Fig 11 14 LDH-activity in international units (U) determined by the automati Technicon method invol ing NAD fluorimetric deter mination For further legends see Fig 11 6

11 15 Glutami -Oxaloaceti transaminase (GOT EC 2 6 1 1) activity i international units (U) determined by the automati Technicon method involving NAD fluori metri determination For further legends see Fig 11 6

Fig 11 16 Glutamic-Pyruvi transaminase (GPT EC 2 6 1.2) activity in i ternational units (U) determined by the automati Technicon method invol ing NAD fluorimetri determination For further legends see Fig 11 6

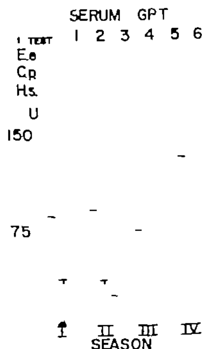


Fig. 11.16

IV MALIC DEHYDROGENASE MDH EC 1 1 1 37

Methods

Determination of the activity of the enzyme The general principles for determination of dehydrogenase activity have been discussed above The reaction mixture in the spectrophotometric measurement was as follows:

10 µl homogenate supernatant

2 4 ml 0 1M Glycine-buffer pH 9 8

0 3 ml 0 1M Na malat (B D H or Sigma)

0.3 ml 4 44 mg/ml NAD (Sigma)

The reaction was started by adding the homogenat supernatant to the est of the reaction mixture

Determination of the relative activity of the isoenzymes The technique was exactly the same as the technique previously described for LDH-isoenzymes The upper half of the starch-gel after lectrophoresis was incubated with the following staining solution:

200 ml 0 2M Tris-buffer pH 8 6

6 ml 2M Na malate

2 4 ml 30 mg/ml NAD

4 ml 10 mg/ml Nitro-BT

1 ml 5 mg/ml PMS

The evaluation of the isoenzyme-bands was the same as for LDH-isoenzymes.

Results

Heart See Fig. 11 17

Guinea-pig heart has greater MDH-activity than hedgehog heart during three seasons (I III IV)

Liver See Fig. 11 18

There are no significant differences between the activity of hedgehog liver MDH and that of guinea-pig

White fat See Fig 11 19

During autumn (I) winter (II) and spring (III) the act ity of hedgehog MDH i higher than that of guinea-pig.

Brown fat See Fig 11 20

The activities of MDH-isoenzymes from the heart of hedgehog and the guinea-pig (Fig 11 21) There are small signifi ant changes of the relative act ity of mitochondrial (M-MDH) and cytoplasmatic (C MDH) MDH from hedgehog heart Between hibernation (II) and spring (III) C MDH increase significantly and betwee spring (III) and summer (IV) C MDH decreases

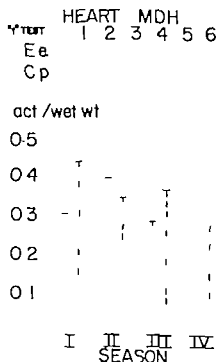


Fig. 11.17

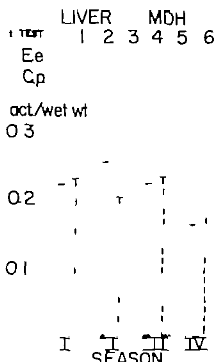


Fig. 11.18

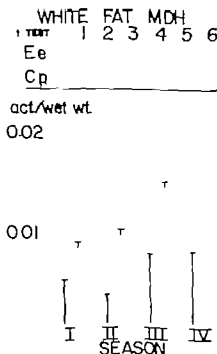


Fig. 11.19

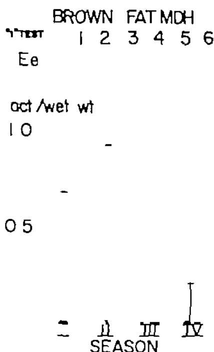


Fig. 11.20

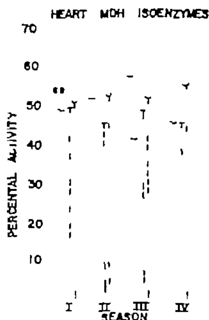


Fig 11.21

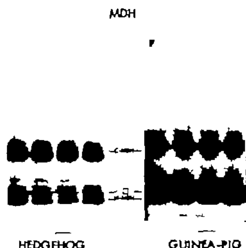


Fig 11 22

and M-MDH increases ignificantly

The MDH-isoenzymes from guinea-pig heart do not hange significantly during the year

Discussion

There is one common feature of the hanges of activity of MDH i heart liver and brown fat from hedgehog the activity of MDH has maximum value during hibernation Thi argues in favor of either an aerobi metabolism or an increased capacity of gluconeogenesi during hibernation Burlington and Klain (1967) have shown an increased capacity for gluconeogenesi i tissues of hibernating Citellus tridecemlineatus Whitten and Kl i (1968a) observed an

Fig 11 17 MDH-activity i units described i the text For further legends see Fig 11 6

Fig 11 18 For legends see Fig 11 17

Fig 11 19 For legends see Fig 11 17

Fig 11 20 For legends see Fig 11 17

Fig. 11 21 Percental activity of the MDH-isoenzymes of heart Cytoplasmi (C MDH) ⊤ Mitochondrial (M-MDH) Y For further legends see Fig 11 17

Fig 11 22 Electrophor tic separation of the isoenzymes f MDH from the myocardium of hibernating hedgehog and guinea-pig from winter

increased incorporation of alanine into hepatic glycogen during hibernation i *Citellus tridecemlineatus*. These studies support the concept of increased capacity of gluconeogenesis during hibernation i   lices of kidney cortex. Burlington (1966) has also shown that the capacity for gluconeogenesis increases during cold exposure both i rats and i hamsters. Rebel *et* l (1960) observed an increased capacity of C^{14}-ac tat incorporation into glycogen in myocardium brown fat and kidney of hibernating C. citellus compared to the non-hibernating state.

The concentration of glycogen from hedgehog heart and liver increased during the hibernation i this study (Chapter 8). Thi points to increased gluconeogenesi during hibernation. It has lso been shown that the glycogen content of fasting animals increases (Adrouny 1969).

As South and House (1967) pointed out there is insufficient information to evaluat the results of Rebel t al. (1960).

Acetat can be incorporated into glycogen via malate-NADP-oxidoreductase (EC 1 1 1 40) oxaloacetate decarboxylase (EC 4 1 1 3) or phosphoenolpyruvate carboxylase (EC 4 1 1.32) (Utter and Kurahashi 1954). If the two latter enzymes ar of great importanc in thi process the activity of MDH should also increase during the hibernation. Whitten and Klai (1968b) have studied the activity of malate-NADP-oxidoreductase from the liver of hibernating and non-hibernating ground squirrels (*Citellus tridecemlineatus*) and they have found that the activity was considerably lower during hibernation than during June and September.

Rognstad and Katz (1970) have shown that malate and oxaloacetat have great importanc in the gluconeogenesi of the rat kidney cortex. From thei values it i evident that MDH has twi   the capacity of phosphoenolpyruvate carboxylase. Thi ratio is calculated from the carbon flow from pyruvate i gluconeogenesis. The results of Rognstad and Katz (1970) imply that MDH is involved i the gluconeogenesis from pyruvate acetat and malate i the tissues studied. Galster and Morrison (1970) eported some interesting data about the glycogen content of the liver and heart of the ground squirrel (*Citellus undulatus*). In the beginning of each period of hibernation after an arousal period the glycogen ontent was relatively high but it then declines until next arousal. The glycogen content of the heart was doubled and constant during the winter. Galster and Morrison (1970) concluded that gluconeogenesis i hibernating *Citellus undulatus* was not sufficient to counteract glycogenolysis. Tashima t al. (1970) proposed from their studies of C^{14}-glucose utilization in *C. lateralis*, that during hibernation glycolytic intermediates ar specifi lly blocked from entering the Krebs cycl . Zimny and Moreland (1968) found that the activity of succinic dehydrogenase from the heart of hibernating *C. tridecemlineatus* decreased to 60% of the non-hibernating value but Chaffee *et* l (1966) found an increased oxidation of succinate by li er mitochondri from hibernating *Citellus lateralis*. Li *et al.* (1969) have also observed a decreased respiratory rat i the liver of hibernati g ground squirrel (*C. tridecemlineatus*) and hamsters (*Mesocricetus auratus*). I contrast to these results Frehn (1966) has found an increased succinate-ADP espiration i liver mitochondria from

hibernating chipmunks (Tamias) compared to non-hibernating hipmunks and a decreased succinate-ADP espiration in liver mitochondria from hibernating hamsters compared to non-hibernating hamsters. Liu et al (1969) suggest that the different responses of succinate respiratory activity to hibernation depend on species differences Decreased activity of the Krebs cycle during hibernation has been found in Citellus (Zimney and Moreland 1968) (Tashima t al 1970) and in Mesocricetus (Liu et al 1969) but Chaffee et l (1961) Denyes and Hassett (1960) Denyes and Carter (1961) South (1960 a and b) and Boumber and Denyes (1963) have ll observed an increased capacity of oxidative metabolism i cold-acclimated and hibernating hamsters compared to non-hibernating Increased activity of the Krebs cycl during hibernation has been found in Tamias (Frehn 1966) Citellus (Chaffee t al 1966) and Erinaceus (activity of MDH from the heart liver and brown fat Figs 11 17 11 18 11 20) There may be differences i the species season method and temperature used whi h may expl in the different e-sults. Further tudies are necessary on several species in order to solve these problems.

The isoenzymes of MDH: Cytoplasmi MDH (C-MDH) and Mitochondrial MDH (M-MDH) from the heart do not hange significantly during the year (Fig 11 21) except the small changes of the isoenzymes of the hedgehog These small changes of C MDH and M-MDH contrast with Moreland (1962) conclusion that the number of mitochondria increases during the hibernation of ground squirrel Citellus tridecemlineatus Zimny and Moreland (1968) correlated this increase of mitochondri to an increased fatty acid oxidation Poche (1959) found that the number of cristae increased but the number of mitochondri was constant during the hibernation of the fat dormouse (Glis glis) (See Chapter 15)

The probabl importance of equal energies of activation of the two isoenzymes for cold resistanc has been proposed by Paulsrud t al (1970) They found that C MDH and M-MDH from the brown fat and the liver of the bat (Eptesicus fuscus) have equal energies of activation (15 2 and 14 9 kcal/M) The heat of activation of the mitochond-i l-MDH was higher than that of the cytoplasmati MDH both from the brown fat (16 7 > 13 1 kcal/M) and the liver (17 0 12 3 kcal/M) of the rat They could corre-late this low value for the energy of activation of the cytoplasmati -MDH of the rat with a high electrophoretic mobility I addition to this Kitto and Wilson (1966) found that C-MDH has lower lectrophoreti mobilities from birds capabl of natural hiberna-tion As is seen from Fig. 11 22 there are no differences between the lectrophoreti mobilities of C MDH from the guinea-pig and the hedgehog but other esults (Olsson 1971) show that ther is lower lectrophoreti mobility of C MDH from the bats (Nyctalus noctul and Vespertili discolor) compared to that from the guinea-pig Summarizing these esults the theory of Paulsrud et al (1970) can be relevant for bats but not for hedgehog on the basis of lectrophoreti mobility

Differences between temperature optima for heart MDH from hedgehog and guinea-pigs have been shown (Olsson 1971) The temperature optima of hedgehogs and bats (45° C) li between that of poikilothermic animal (40° C) and that of homeothermic animal the guinea-pigs (50° C) There are no changes of MDH whether the hedgehog is hibernating or not neither are there any great differences in the energy of activation from the heart of guinea-pig hedgehog or bat These results show that there have been adaptive changes of MDH i the evolution of homeothermal and hibernating mammals and thi is quite in agreement with Johansson s (1969b) observations on LDH and holinesterase

During the hibernation aerobic metabolism in hedgehog white fat is significantly reduced as shown in the decrease of MDH activity

Brown fat has a high capacity for aerobic metabolism and thus exhibits a high activity of MDH This activity i higher during hibernation and posthibernation. During these periods the brown fat is hyperactive i the arousal processes Hedgehog brown fat has thermogenic effect during arousal even i a warm environment (Edwards and Munday 1969a) Drahkoczy and Lyman (1965) and Edwards and Munday (1969a) have observed that brown fat is active during hibernation. This is i agreement with the high activity of MDH during hibernation and posthibernation Edwards and Munday (1969a) noticed that it was impossibl to relat the activity of the brown fat during hibernation with heat production inc the temperature of brown fat and other areas could not be recorded without initiating arousals. Hibernation i naturally interrupted by several arousals (Kristoffersson and Soivio 1964 Galster and Morrison 1970)

I this study the period of posthibernation is very lose to hibernation as some of the hedgehogs from the posthibernation period i fact were still hibernating (see above Chapter 2)

V GLUCOSE-6-PHOSPHATE DEHYDROGENASE G-6-PDH EC 1 1 1 49

Methods

The general principles for the determination of dehydrogenase activity have been discussed above The reaction mixture I the spectrophotometric measurement was as follows:

100 µl supernatant of the homogenat

2 6 ml 0 1M Tris-Buffer pH 8 0

0 1 ml 0 03M Glucose-6-phosphat (Sigma)

0 1 ml 0 01M NADP (Sigma)

0 2 ml 0 15M $MgCl_2$

The reaction was started by adding the supernatant of the homogenat to the rest of the reaction mixture

Results

Heart See Fig. 11 23

The activity from hedgehog heart is twice as high as that from guinea-pig heart

Liver See Fig. 11 24

The activity from hedgehog liver is twic that of guinea-pig liver

White fat See Fig 11 25

The activity of G-6-PDH from the guinea-pig whit fat is almost ten times that of the hedgehog

Brown fat See Fig. 11 26

Discussion

The activity of G-6-PDH from hedgehog heart and liver decreases after hibernation and seems to increase just before hibernation T leologically the activity of G-6-PDH appears not to be as important for hedgehog heart as for that of the guinea-pig sinc the activity is higher in guinea-pig heart Furthermore the activity of G-6-PDH I 8 times as high in liver as I heart of the hedgehog and only twice as high in the same organs of the guinea-pig.

The ground squirrel (Citellus undulatus) has 18 and the rat 4 times higher G-6-PDH activity I liver than in heart (Hannon and V ughan 1961) Thi suggests that G-6-PDH has relatively greater importanc for the liver of the hibernator than for liver of the non-hibernator and vice versa for the heart

Hannon and Vaughan (1961) lso found small increase of the activity of G-6-PDH from the heart of hibernati g ground squirr l Citellus undulatus, which compared to non-hibernating ground squirr ls supports the above mentioned changes for the heart of the

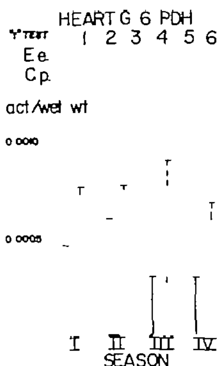


Fig 11.23



Fig. 11.24

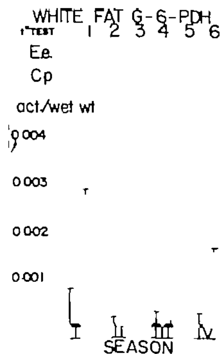


Fig. 11.25

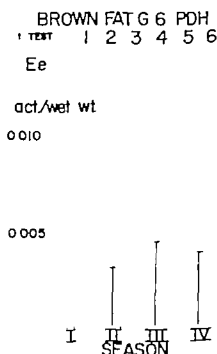


Fig 11.26

hedgehog Hawley (1968) has shown that the oxygen uptake in vitro t 35° C is greater in the heart of the ground squirrel (C tridecemlineatus) when hibernating than when not hibernating in the presence of G-6-P NAD(H) of NADP(H) and F This shows that the pentose-shunt has greater importance during hibernation than i the aroused rat which is i agreement with the high activity of G-6-PDH from the heart and the liver of the hibernating hedgehog Hawley work (1968) suggests that the hibernating heart is more dependent on fatty acid catabolism than the non-hibernating heart

G-6-PDH has great importance for the synthesi of fatty acids and steroids as $NADPH_2$ is formed at the oxidation of G-6-P (Tepperman and T pperman 1958 1961 South and House 1967) It is important in thi discussion to remember that $NADPH_2$ also can be formed by other processes (Lowenstein 1961 Flatt and Ball 1964 Pande et l 1964 and Kornacker and Ball 1965)

Forsberg and Sarajas (1955) found higher total liver lipid content and an increased incorporation of glucose into the liver lipids i hibernating hedgehogs (Chapter 9) This is in agreement with the high G-6-PDH activity of the liver of the hibernating hedgehog but this is not supported by Hannon and Vaughan (1961) who found that the activity of G-6-PDH from the liver of ground squirrel (Citellus undulatus) decreased during the hibernation. Whitten and Klai (1968b) lso eported a decreased incorporation of glucose and acetate into lipids and decrease of G-6-PDH activity i the liver of hibernating ground squirrels (Citellus tridecemlineatus) It i quite possibl that there ar great species differences i the lipogenesis during hibernation. Tashima t al (1970) uggest from their studies of C^{14} incorporation that lipogenesi is profoundly depressed in the heart the liver the white and brown fat of ground squirrels (Citellus lateralis) during hibernation. Denyes and Carter (1961) showed that ther i decrease of lipogenesis in the liver of hamsters (Mesocricetus) during the hibernation Baumber and Denyes (1963) found an increase of lipogenesis i the epididymal fat during hibernation i hamsters (Mesocricetus) It is evident that there is lipogeni differenc between these two organs of hamsters (Mesocricetus) It seems as it the liver and the whit fat of the hedgehog behave i an opposit way Lipogenesi i hedgehog white fat may be decreasing duri g hibernation as is seen from the striking decrease of the activity of G-6-PDH It is well-known that lipogenesi

---

Fig. 11 23.G-6-PDH-acti ity i units described i the text For further legends see Fig 11 6

Fig. 11 24 For legends see Fig 11 23

Fig 11 25 For legends see Fig 11 23.

Fig 11 26 For legends see Fig 11 23

is high during prehibernation (Kayser 1961 Smalley and Dryer 1967) Thus thi decrease in the activity of G–6–PDH from the white fat between prehibernation and hibernation can be seen as consequenc of the high lipogeni activity during the prehibernation period

The changes in activity of G–6–PDH from the brown fat of the hedgehog are almost the same as from the white fat with the exception that the activity from the brown fat increased even after hibernation Thi indicates that the lipogenesis i this tissue starts almost at once after hibernation (Chapter 15) Tashima et al (1970) found an accelerated incorporation of glucose into total lipids and especially glyceride–glycerol i the brown fat during the first hour of arousal in ground squirr l (Citellus lateralis)

VI α-GLYCEROPHOSPHATE DEHYDROGENASE α-GPDH EC 1 1 1 8

Methods

The general principles for determination of dehydrogenase activity have lready been discussed

The reaction mixture in the spectrophotometri measurement was as follows:

100 µl homogenate supernatant

2 4 ml 0 1M Glycine-buffer pH 9 22

0 3 ml 0 1M α-Glycerolphosphate (Sigma)

0.3 ml 4 44 mg/ml NAD (Sigma)

The reaction was started by adding the supernatant of the homogenate to the rest of the reaction mixture

Results

Heart See Fig 11 27

The activity of α-GPDH from hedgehog heart is significantly higher than that of the guinea-pig during all seasons except the autumn (I)

Liver See Fig 11 28

The activity of α-GPDH from guinea-pig liver is significantly higher than that of the hedgehog except during the autumn (I)

White fat See Fig 11 29

During winter and spring the activity of α-GPDH from whit fat is higher from guinea-pig than from hedgehog

Brown fat See Fig 11 30

Discussion

α-GPDH from hedgehog heart has significantly higher activity than from guinea-pig heart except for autumn Bücher and Klingenberg (1958) and Sacktor (1959) showed that ther are both extra- and intramitochondri l forms of α-GPDH The intramitochondrial form oxidizes α-GP to dihydroxyac tonephosphat which the extramitochondrial form reduces to α-GP Chaffee et al (1964) Chaffee et l (1966) and Dryer and Pauleryd (1966) have emphasized the importance of thi process for the gain of energy from glycerol from f t catabolism. During hydrolysis of fats glycerol I formed which is phosphorylated to α-GP whi h then can be utilized I glycolysis (Chaffee et l 1964 Dryer and Pauleryd 1966) by oxidation

The low activity of α-GPDH from hedgehog heart during autumn can be correlated to the low fat atabolism during that season The ignificantly higher activity of α-GPDH from

HEART ALPHA GPDH

t-TEST 1 2 3 4 5 6

Ee

Cp

act./wet wt

0.010

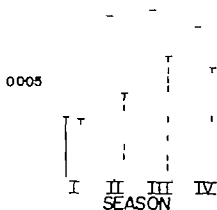


Fig. 11.27

LIVER ALPHA GPDH

t-TEST 1 2 3 4 5 6

Ee

Cp

act./wet wt.

0.01

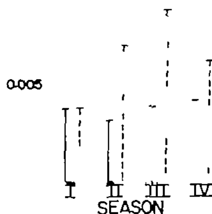


Fig. 11.28

WHITE FAT ALPHA GPDH

t-TEST 1 2 3 4 5 6

Ee

Cp

act/wet wt

0.002

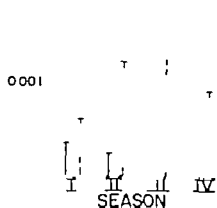


Fig. 11.29

BROWN FAT ALPHA GPDH

t-TEST 1 2 3 4 5 6

Ee

act./wet wt.

0.02

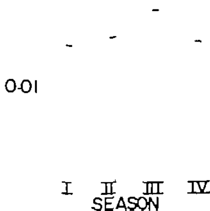


Fig. 11.30

hedgehog heart and the increase in activity during hibernation and the decrease after the spring can imply that hedgehog heart uses glycerol phosphate as an energy pool to a greater extent during hibernation i comparison with the prehibernatory state and guinea-pig heart

The activity of α-GPDH is higher i the li er than i the heart of the guinea-pig The increase in activity of α-GPDH from hedgehog liver between hibernation and post-hibernation is in agreement with the high lipolysis seen during the spring The low activity of α-GPDH during hibernation supports the results of Chaffee et al (1966) who found a low α-GPDH activity i the liver mitochondria of hibernating ground squirrels (Citellus lateralis)

The significant increase of the activity of α-GPDH from hedgehog white fat between winter and spring can be an expression of the high lipolytic activity during the spring The serum glyceride-glycerol concentration of the hibernating hedgehog was low but it was higher when arousing (Konttinen et al 1964) The same tendency as seen in whit fat is quite obvious i the changes of α-GPDH from the hedgehog brown fat I this tissue there i an increase in activity before spring and a decrease after spring This is correlated to a high rate of lipolysis during arousals from hibernation and the decrease i lipolysis rate after arousals. Joel (1965) found a 50% decrease in lipid concentration i recently aroused ground squirrels (Citellus tridecemlineatus) i comparison with the concentration just before arousal This great release of lipids and glycerol can be utilized by other organs.

Hardman and Hull (1970) concluded from their studies on newborn rabbits "that brown adipose tissue releases ignificant amounts of fatty acid and glycerol into the irculation and that this contribution is greatly increased with noradrenalin infusion and presumably cold exposure and that brown adipose tissue depleted of fat produces heat by drawing free fatty acids as well as glucoses from the circulation Horwitz and Nelson (1968) agree with these conclusions. Prusiner et al (1968) proposed that most of the energy for nonshivering thermogenesis comes from fatty acid oxidation I this connection it is important not to overlook the calorigeni potential whi h arises from the oxidation of

---

Fig 11 27 α-GPDH-activity i units described i the text For further legends see Fig 11 6

Fig 11 28 For legends see Fig 11 27

Fig. 11 29 For legends see Fig. 11 27

Fig 11 30 For legends see Fig. 11 27

α-GP (Green 1936 Bücher and Klingenberg 1958 Estabrook and Sacktor 1958 Smith 1964) Brown fat has the greatest activity of α-GPDH in the organs studied whi h supports the observation by Chaffee et al (1966) that i ground squirrel (Citellus lateralis) as in hamsters (Mesocricetus) (Chaffee et al 1961) the respiration of mitochondria on α-GP was low i systems from the liver but relatively high i those from brown fat

Prusiner et al (1970) reviewed the mechanisms controlling oxidative metabolism i brown fat and mentioned that α-GP as a substrat as well as succinate and fatty acids + carnitine stimulated oxidation both in mitochondria and in cells of brown fat Thus α-GP may be important for brown fat both as a trigger substanc and as c lorigeni substance As mentioned above the activity of α-GPDH is high in brown fat and thus brown fat can oxidize α-GP

Chaffee t l (1966) found a maximum for α-GPDH activity from brown fat during hibernation of ground squirrel (Citellus lateralis) The activity of α-GPDH from hedgehog brown fat had its maximum value during spring the arousal season

VII SUMMARIZING DISCUSSION

The seasonal changes of the activities of the four dehydrogenases LDH MDH G-6-PDH and α-GPDH from heart liver whit and brown fat of hedgehog have one common feature In ll organs except whit fat all enzymes have maximal activity during hibernation However G-6-PDH in brown fat and α-GPDH in brown fat and α-GPDH in liver ar exceptions Seasonal hanges in activities of these four enzymes from the same organs of guinea-pig are quite different from that of hedgehog Only α-GPDH from hedgehog and guinea-pig heart show imilar changes.

The activities of LDH and α-GPDH in heart and G-6-PDH in liver from hedgehog are significantly higher than the corresponding activities from guinea-pig On the other hand the activities of MDH and G-6-PDH i heart α-GPDH i liver and MDH G-6-PDH and α-GPDH i whit fat from guinea-pig ar significantly higher than the activities from hedgehog These results may indicate the following differences between the hedgehog and guinea-pig:

1 a higher anaerobi capacity for heart and liver of hedgehog than guinea-pig (see discussion below and in the section on LDH above)

2 higher capacity for the pentose-shunt in hedgehog liver and i guinea-pig heart and white fat than other tissues examined (see discussion in the section on G-6-PDH)

3 higher capacity for glycerol metabolism in hedgehog heart and i liver and whit fat of guinea-pig than in other tissues examined (se discussion i the section on α-GDPH)

It may not be meaningful to compare seasonal changes of enzyme activities from the hedgehog hibernator with those from the guinea-pig non-hibernator since the two species did not live under identical environmental conditions i this study (see Chapter 2 above) In addition the two species belong to different mammalian orders <u>Insectivora</u> and <u>Rodentia</u>

Some information about the ratio of anaerobic metabolism may be obtained by the enzyme activity quotient Q LDH/MDH which was proposed by Bücher and Klingenberg (1958) When applying the quotient to hedgehog and guinea-pig organs (see Table 11 0) it i evident that the hearts of both species have dominant aerobic metabolism as does guinea-pig liver This is i agreement with van den Hende <u>et al</u> (1968) who have studied Q LDH/MDH in the same organs from pigs It is remarkabl that the quotient from hedgehog liver is so high. This supports the observation that hibernators have high anaerobi capacity (see section on LDH above) The quotient is lso littl higher from hedgehog white fat than from guinea-pig white fat Hedgehog brown adipose tissue has an extremely low quotient and it i well known that this organ has very high aerobi metabolism (Lindberg ed 1970)

It has been stated that hibernators ar more capabl of anaerobic metabolism than non-hibernators This study lends support to thi opinion. One indication i that the quotient of LDH/MDH for liver and whit fat are higher i hedgehog than in guinea-pig irrespect of season Another indication is the higher activity of LDH-H from guinea-pig i

Table 11.1

Ratio of the mean activities of LDH and MDH (Q LDH/MDH)

		I	II	III	IV
Heart	Hedgehog	0 41	0 35	0 46	0 54
Heart	Guinea-pig	0 27	0 31	0 31	0 30
Liver	Hedgehog	1 6	1 5	1 3	1 6
Liver	Guinea-pig	0 33	0 34	0 35	0 24
Whit fat	Hedgehog	1 2	1 7	1 5	1 4
Whit fat	Guinea-pig	0.56	0 91	0 93	1 0
Brown fat	Hedgehog	0 13	0 097	0 12	0 25

compared to hedgehog The latter conclusion is questionabl because the LDH-isoenzymes from gui-nea-pig heart do not show a strict binomial distribution

The lower activity of LDH-H from heart of the hibernating hedgehog as compared with the non-hibernating hedgehog should indicate decreased capacity for aerobic metabolism during hibernation However the LDH/MDH quotients do not differ between hibernating and non-hibernating hedgehogs

The increased activity of MDH during hibernation of the hedgehog can either be an expression for increased aerobi capacity or increased capacity of gluconeogenesis which is discussed in the section on MDH I light of the results from LDH and Q LDH/MDH It is probabl that the increased acti ity of MDH represents an increased capacity for gluconeogenesis in hedgehog heart and liver On the other hand brown adipose tissue with a very high aerobic metabolism increased its aerobi capacity further during hibernation by increasing MDH-activity White adipose tissue with a dominant anaerobi metabolism reacted in the opposite way during hibernation

The importanc of the pentose-shunt is to some extent the synthesis of $NADPH_2$, which is discussed in the section on G-6-PDH The activity of G-6-PDH which i the first enzyme in the pentose-shunt decreases after hibernation i hedgehog heart and liver and increases slightly before hibernation Thi seems t be an expression for high lipogenesi i heart during hibernation and lower one after hibernation. Lipogenesi i whit and brown fat is maximal i autumn as is seen from the maximal values of G-6-PDH activity i autumn I addition the activity of G-6-PDH i brown fat increases after hibernation.

The activity of α-GPDH is eflection of glycerol turnover and thus the rat of metabolism of lipids It appears that hedgehog heart has a high apacity for the turnover of lipids especially during hibernation and spring Thi i supported by the high activity of G-6-PDH i

heart during hibernation The adipose tissues both white and brown show maximal α-GPDH-activity during spring the arousal season

The most interesting result from the changes of solubl proteins I the maximal values during hibernation and posthibernation I brown fat of hedgehog This may be correlated to the large number of mitochondria during hibernation and posthibernation (see Chapter 15) The mitochondrial changes are correlated to the high aerobic metabolism I this tissue

Table 11 1

Ratio of the mean activities of LDH and MDH (Q LDH/MDH)

		I	II	III	IV
Heart	Hedgehog	0 41	0 35	0 46	0.54
Heart	Guinea-pig	0 27	0 31	0 31	0 30
Liver	Hedgehog	1 6	1 5	1 3	1 6
Liver	Guinea-pig	0.33	0 34	0 35	0 24
White fat	Hedgehog	1 2	1 7	1.5	1 4
Whit fat	Guinea-pig	0.56	0 91	0 93	1 0
Brown fat	Hedgehog	0 13	0 097	0 12	0 25

compared to hedgehog The latter conclusion i questionabl because the LDH-isoenzymes from guinea-pig heart do not show a strict binomial distribution

The lower activity of LDH-H from heart of the hibernating hedgehog as compared with the non-hibernating hedgehog should indicate decreased capacity for aerobi metabolism during hibernation However the LDH/MDH quotients do not differ between hibernating and non-hibernating hedgehogs

The increased activity of MDH during hibernation of the hedgehog can either be an expression for increased aerobi capacity or increased capacity of gluconeogenesis which is discussed in the section on MDH I light of the results from LDH and Q LDH/MDH It is probabl that the increased activity of MDH represents an increased capacity for gluconeogenesi in hedgehog heart and liver On the other hand brown adipose tissue with a very high aerobic metabolism increased its aerobi apacity further during hibernation by increasing MDH-activity White adipose tissue with dominant anaerobi metabolism reacted in the opposite way during hibernation

The importance of the pentose-shunt is to some extent the synthesi of $NADPH_2$ which i discussed i the section on G-6-PDH The activity of G-6-PDH which is the first enzyme i the pentose-shunt decreases after hibernation i hedgehog heart and liver and increases slightly before hibernation. This seems to be an expression for high lipogenesi in heart during hibernation and lower one after hibernation Lipogenesis in whit and brown fat i maximal i autumn as is seen from the maximal values of G-6-PDH activity i autumn I addition the acti ity of G-6-PDH in brown fat increases after hibernation

The activity of α-GPDH is reflection of glycerol turnover and thus the rate of metabolism of lipids It appears that hedgehog heart has high capacity for the turnover of lipids especi lly during hibernation and spring Thi is supported by the high acti ity of G-6-PDH i

the principles of Axelrod (1962) The activities of COMT and PNMT were expressed I counts per 10 ml product formed per mg protei per 60 min incubation Control analysis showed no decrease in COMT MAO and PNMT activity after storage at 70^{o} C up to 2 months

The protein content of the tissue preparations was determined by the method of Lowry *et al* (1951) All analyses were run as duplicates

The biosynthesis of epinephrine from norepinephrine and the catabolI pathway of both compounds is given I Figure 12 1 See further Chapter 2 General Methods and Materials

Results

The results are shown in Figures 12 2–12 10

Guinea-pig The concentration of NE in the left ventricle of the guinea-pig heart showed only modest seasonal variations The level was essentially constant during all periods except summer when the concentration had increased by more than 65 per ent The activity of MAO I the heart was maximal in the spring and that of COMT I the wi ter lthough high COMT values wer also found during spring Both catecholamine-catabolizing enzymes showed minimum activity at fall The acti ity of PNMT was generally low and showed little seasonal variation.

In the adrenal glands the oncentration of E was onsiderably higher than that of NE. The level of NE showed steady increase from fall to 100 per cent higher value during summer whereas E was highest during f ll and summer and lower during winter and spring The adrenals had their largest absolut amounts of both catecholamines during summer; the ratio of E/NE on the other hand was maximal during fall The activities of MAO and COMT were highest during winter and the lowest values of these enzymes wer ecorded at fall The PNMT activity also had its maximum I the winter but its minimum during the summer period

Hedgehog In the hedgehog the mean values for the whol year of NE in the heart left ventricle was only about one third of that of the guinea-pig The concentration exhibited marked seasonal changes from a low value during fall with subsequent tendency to continuous increase to maximum level during summer when the concentration was 4 times higher than in the fall The activities of both MAO and COMT wer highest during spring and low activities were registered during summer and f ll As I the guinea-pig PNMT activity was low and seasonal variations were essentially absent Sometimes no PNMT activity at ll could be found in the heart

The concentration of E was much higher than that of NE in the adrenal glands also I the hedgehog The E level was remarkably constant throughout the year whereas NE like E I the guinea-pig was high during fall and summer and low during winter and spring Agai the highest absolute concentrations of both catecholamines were found during the summer

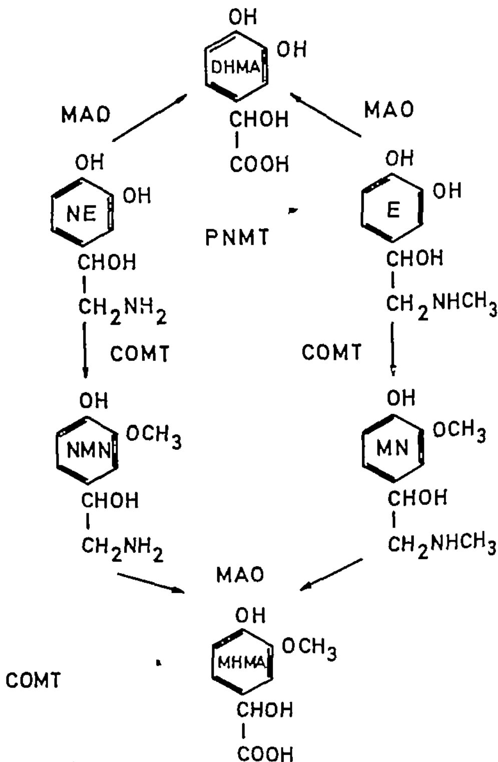

OH
OH
DHMA
CHOH
COOH
MAO
MAO
OH
OH
NE
CHOH
CH_2NH_2
PNMT
OH
OH
E
CHOH
CH_2NHCH_3
COMT
COMT
OH
OCH_3
NMN
CHOH
CH_2NH_2
OH
OCH_3
MN
CHOH
CH_2NHCH_3
MAO
OH
OCH_3
MHMA
CHOH
COOH
COMT

Fig 12 1

period. The E/NE rati which was characteristically much lower in the hedgehog than in the guinea-pig was maximal during winter The highest values of MAO and COMT were found in the winter and spring periods respectively; the lowest values were measured at fall and summer The PNMT activity decreased continuously from high levels during fall to mi imum i the summer period.

Comparison between guinea-pig and hedgehog The results showed that the concentration of NE in the hedgehog left ventricl was only about one third of that found in the guinea-pig In the adrenals however the NE concentration was more than 10 times higher in the hedgehog. Also the concentration of E was considerably higher i the latter animal Nevertheless the guinea-pig had an adrenal E/NE ratio that was about 4 times higher than i the hedgehog

The concentration of catecholamines in the heart as well as in the adrenals were maximal during summer in both species of animals However the minimum values were all found at different periods and the patterns of seasonal variation were different i the two animals.

Low values of the two atecholamine-catabolizing enzymes were seen both in the heart and i the adrenal during fall In the adrenals of the hedgehog the COMT activity was high both during winter and spring whereas in the guinea-pig high level was found only in the winter The same was true for the maximal MAO activity

In the heart the MAO acti ity was higher in the guinea-pig than in the hedgehog. The activity of COMT on the other hand was considerably higher i the hedgehog In the adrenals both COMT and PNMT activities were highest in the hedgehog

If the activity of COMT i calculated in terms of nmoles of product formed tentative comparison can be made with the MAO activity in the same tissue Thus in the hedgehog heart the MAO activity was about 50 times higher than that of COMT Cardiac MAO activity i the gui nea-pig was about 1 000 times higher than that of the COMT activity I the adrenal of both animal the MAO activity was about 70 times higher than the COMT activity

I both the guinea-pig and hedgehog the maximum values for cardiac NE were found i the summer period, thi corresponded to low activity of COMT On the other hand low concentrations of NE i the heart wer recorded during fall when MAO activity was mi imal

Fig 12 1 Biosynthesi of epinephrine (E) from norepinephrine (NE) by phenylethanolamine-N-methyltransferase (PNMT) and catabolism of E and NE by means of catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) DHMA = 3 4-dihydroxymandelic acid MHMA = 3-methoxy-4-hydroxymandeli acid NMN normethanephrine MN = metanephrine

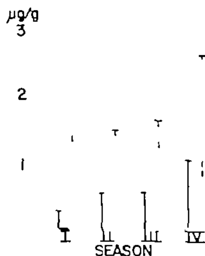


Fig 12 2

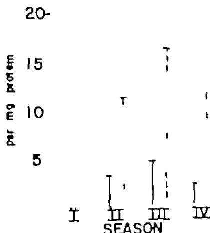


Fig. 12 3

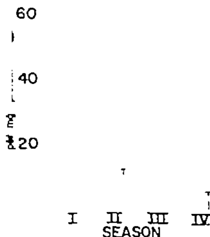


Fig. 12 4

The catecholamine concentration in the adrenals was maximal at summer in both species of animals and agai this corresponded to low COMT activity Contrary to the ituation in the heart low catecholamine level in the adrenal were in both animal seen t o period (winter) when the MAO acti ity was found to be maximal

The PNMT acti ity measured i the adrenals of the guinea-pig and hedgehog was mi imal in the summer when the highest E values wer found i both animals. A maximal rati of E/NE which was obtained during f ll in the guinea-pig and during wi ter i the hedgehog corresponded to high activities of PNMT

Discussion

Fluorescence histochemistry using the formaldehyde condensation technique of Falck and Hillarp has demonstrated that cardiac NE is located i a system of adrenergic sympathetic nerves arranged in a typi al autonomic ground-plexus (se Ni lsen and Owman 1968) I the guinea-pig as in other species of non-hibernating mammal the adrenergi nerv terminals in the heart ventricles ar haracteristically distributed around blood vessels down to the smallest arterioles as well as in lose relation t the cardiac muscl fibers (Angelakos et al 1963 Dahlström et al 1965 Ehinger et al 1967 Jacobowitz et al 1967 Nielsen and Owman 1967 Ehinger et al 1968 Ni lsen and Owman 1968 Angelakos et al 1971) NE is present i adrenergic nerves lso i the heart of hibernators (Nielsen and Owman 1965 1968 Angelakos et al 1971) but the distribution of the axons i the ventri les i markedly different from that found i non-hibernators (Ni lsen and Owman 1965 1968) Thus the bulk of nerve terminals enclose the blood vessels whereas few if any terminal are seen i relation to the myocardial muscl fibers The presence of adrenergic nerve terminal i the

Fig 12 2 Heart norepinephrine i µg/g. Seasons refer to the prehibernating (I) hibernating (II) posthibernating (III) and active non-hibernating (IV) phase See further Fig 2.1 i General Methods and Materials The solid line refers to hedgehogs and the interrupted line to guinea-pigs The number of denot the ignificanc level according to the "t test one means 0 05 P 0 01 and two P 0 01 E refers to the hedgehog (Erinaceus europaeus) and C p to th guinea-pig (Cavi porcellus) 1 indicates the difference between season I and II 2 between I and III 3 between I and IV 4 between II and III 5 between II and IV and 6 between III and IV

Fig 12 3 Heart monoamine oxidase; nmol formed product per mg protei per 60 mi incubation See Fig 12 2 for legend

Fig 12 4 Heart catechol O-methyltransferase; counts per 10 mi produced formed per mg prot i per 60 mi incubation See Fig 12 2 for legend

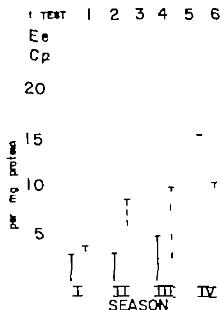


Fig. 12.5

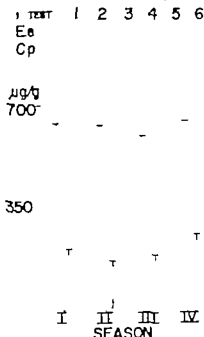


Fig. 12.6

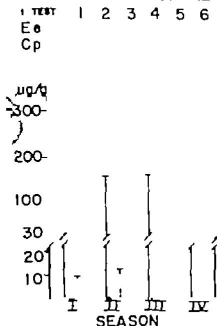


Fig. 12.7

myocardium proper is almost impossible to exclude in ordinary preparations which may well explain the inability of Angelakos et al (1971) to confirm the above-mentioned pattern of distribution; the findings by Nielsen and Owman (1968) on the other hand were based upon serial sections of ventricular tissue i which the vascular system was outlined by previous India ink infusion. Moreover the difference in th adrenergic innervation of the ventricles is effected in the results of the fluorometric determinations of the NE concentration i the heart ventricles which in the hedgehog was only one third of that i the guinea-pig a comparison which can be considered relevant because the two animals are of about equal size It has been suggested (Nielsen and Owman 1968) that the difference i the innervation may offer one direct possibility to explai the marked differenc i cardiac susceptibility to lowered temperature in hibernators compared with non-hibernators In accordance with this the sensitivity of the f line heart to hypotherm! i greatly reduced by various factors that exclude the sympathetic nervous influenc on the myocardial beta-receptors (see Nielsen 1968 Falck et al 1972a)

It should be noted that lthough the present figures on the amount of ventricular NE of the hedgehog show marked and lear-cut seasonal variations th mean values as calculated for the active period (summer and fall) do not differ from those of the hibernating and arousal periods (winter and spring) Henc when expressed i these terms the findings are in good agreement with the dat reported by Musacchi et al (1963) and Draskoczy and Lyman (1967) on ground squirrels and by Uuspää (1963a) on hedgehogs that the concentration of cardiac catecholamines is not significantly different i the active and hibernating periods.

Seasonal variations i the concentration of catecholamines were found in the adrenal gland of both guinea-pigs and hedgehogs It is not likely that changes i the adrenal weight (including changes i the weight of the adrenal medulla) have overtly influenced on the pattern of seasonal variations expressed as amine concentration per unit weight of the whol gland It is well established that E and NE in the adrenal medulla are stored in different cells. Thi agrees with the present findings that the variations in the concentration of E and NE seem to occur independently i the present study the concentration of E i the hedgehog adrenals was found to remain essenti lly unchanged throughout the year and thus the higher level found by Suomalainen and Uuspää (1958) and by Uuspää (1963b) during the hibernating period could not be corroborated However the f ll i NE

Fig 12 5 Heart phenylethanolamine-N-methyltransferase See Fig 12 2 12 4 for legend

Fig 12 6 Adrenal epinephrine See Fig 12 2 for legend

Fig 12 7 Adrenal norepinephrine See Fig 12 2 for legend

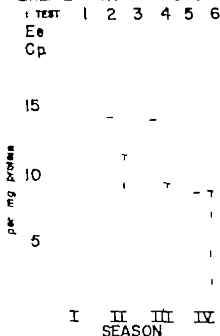


Fig. 12.8

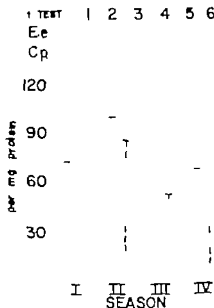


Fig. 12.9

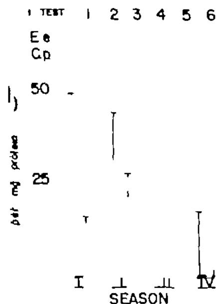


Fig. 12.10

during the winter and spring periods, and hence a maximal E/NE ratio during these periods agrees with the data reported by the above-mentioned authors

No report is available on the seasonal variation of the presently studied enzymes in the guinea-pig and hedgehog Only one investigation deals with seasonal variations of MAO activity in different parts of the brain of the rabbit (Kojima 1966) Maximum values wer found during fall whereas the lowest activity was registered at spring The changes are thus opposite to those see i the heart and adrenals of the guinea-pig and hedgehog

The low summer activity of COMT registered in the heart and adrenals of both species corresponding to maximal catecholamine levels may indicat the presenc of an inc eased amount of NE in the large llular storage pool in combination with an increas in fr NE reaching the receptors in the heart and the circulation from the adrenals This would agree with an enhanced sympathetic tone during the summer period

It is well-known that MAO is responsible for the continuous inactivation of the excess of catecholamines stored i the c lls. Therefore the combination of ml imum MAO acti vity and low catecholamine levels during fall ar onsistent with relatively low turn-over rate of stored catecholamines during thi period

The presence of PNMT activity in the heart reflects the synthesis of E which is usually present in small amounts i the heart There i no evidence from fluorescence histochemi cal studies that E is stored in adrenergi nerves of mammals It seems more reasonabl to assume that E is present i chromaffi cell known to occur i the heart

The low PNMT activity found in the adrenals i combination with high catecholamine con-entrations may be esult of the known substrate-product inhibition of thi enzyme (Fuller and Hunt 1967) Characteristically high adrenal PNMT activity was found concomitant with maximal ratio of E/NE. Thi may effect particularly high synthesis of E from NE

Fig 12 8 Adrenal monoamine oxidase See Fig 12 2 12 3 for legend

Fig 12 9 Adrenal catechol O-methyltransferase See Fig 12 2 12 4 for legend

Fig 12 10 Adrenal phenylethanolamine-N-methyltransferase See Fig 12 2 12 4 for legend

Chapter 13

Sympatheti Nervou Sy tem II Histochemistry

by

Kai C Nielsen and Christer Owman

Methods

The fluorescence microscopi distribution of serotonin (5-HT) and atecholamines was accomplished by the formaldehyde method of Falck and Hillarp (F l k 1962 Fal k et l 1962 Corrodi and Jonsson 1967) The followi g organs were analyzed in the guinea-pig: pituitary and thyroid glands right atrium and apex of the heart vas deferens (mid-way between bladder and testis) and uterus (mid portion of uterine horn) The same tissues together with interscapular brown fat were taken from the hedgehogs

Immediat ly after removal from the animal the preparations were frozen i liquid mixtur of propane and propylene cooled with liquid nitrogen freeze-dried treated with formaldehyde gas according to the tandard procedure embedded in paraffin sectioned serially at 6 μ thi kness and mounted for fluorescence microscopy For further technical det ils see Falck and Owman (1965) and Björklund et al (1972) Under the optic l conditions used the catecholamine fluorophores appear green whereas that of 5-HT is yellow See further Chapter 2 General Methods and Materials

Results and discussion

The formald hyd histofluorescence technique did not eveal any yellow-fluorescent (serotonin-contai ing) structures in any of the organs studied The results ar thus mai ly concerned with the histocheml al demonstration of norepi ephrine i adrenergic (sympatheti) nerves.

Guinea-pig Fluorescenc microscopy of the pituitary gland revealed fairly large number of very slightly fluorescent ll which wer often collected i small groups throughout the anterior lobe The fluorescence colour was greenish-yellow but difficult to define exactly because of the low intensity I the rat adenohypophysi most of the formaldehyde-fluorescent ll have been found to be PAS-positive (Dahlström and Fuxe 1966) A similar greenish-yellow fluorescenc was found i most if not all of the cells onstituting the

pars intermedi On the basis of the conditions under which the ellular fluoresc nc l the pituitary develops and its spectral characteristics in combination with hemic l determinations it has recently been suggested (Håkanson et al 1972) that the fluorophor l due to the presence of tryptophyl-peptide rather than monoamine The intermediate lobe lso contained delicate green-fluorescent nerve terminals without relation to blood vessel Such nerves were also found in f l ly large numbers scattered l the neurohypophysis whos parenchyma was otherwise completely non-fluorescent Cytospectrofluorometric analysi together with stereotaxic operations have indi ated that the pituitary nerves at least in the rat contain dopamine and arise in the hypothalamus (Björklund et l 1970) The fluorescenc microscopic pictur of the guinea-pig pituitary thus resembled that reported for other mammal (Björklund 1968 Björklund and F lck 1969 and b)

The thyroid gland contained a small number of adrenergi nerves most of whi h accompanied blood vessels lthough it could not be excluded that some fibres also ran in close association with the (non-fluorescent) folli ular epithelium. There is evidenc from experiments on mice that activation of the thyroidal sympathetic nerves which originat l the superior cervical ganglia can induc endocytosis of thyroglobuli and release of thyroid hormone through direct effect on alpha receptors l the thyroid follicle cell (Melander et l 1972) The cal itonin-producing cell (C cells) of the thyroid did not fluoresce (cf F l k and Owman 1968)

The fluorescence microscopi distribution of adrenergic nerves l the mammalian heart including that of the guinea-pig has been reported l detail elsewhere (g Dahlström et al 1965 Ehinger et al 1967 Jacobowitz et l 1967 Ni lsen and Owman 1968) Both the atri (Fig 13 1 a) and ventricles (Fig. 13 2 a) received numerous adrenergi nerve terminal whi h innervate both vessel and the myocardium proper The density of the nerve plexus was higher l the atria (Fig 13 1) than in the ventricles (Fig 13 2 a)

The vas deferens (Fig 13 3 a) received an extremely l h adrenergic innervation The varicose nerve terminal losely followed the direction of the smooth muscl cells thus forming prominent ircular layer with an outer longitudinal layer superimposed (see lso F lck et l 1965) The fibres belong to the special system of "short adrenergi neurons whi h arise l adrenergi ganglion ll located l the terminal parts of the hypogastric nerve where it joins the vessels to the accessory genital organs and ramifies (Owman and Sjöstrand 1965 Sjöstrand 1965)

Also the adrenergi innervation f the mammalian uterus has been subject to extensive studies (see Marshall 1970) The guinea-pig uterus (Fig 13 4 a) received moderate number of adrenergi nerves whi h supplied not only the smooth muscl layers but also blood vessels especially those running between the outer longitudinal and the ircular muscl layers The uterine adrenergi nerves too belong to the system of hort adrenergic neurons (Sjöberg 1968c which originate l ganglia at the utero-vaginal junction (Owman and Sjöberg 1966, Ow



Fig. 13.1a



Fig. 13.1b



Fig. 13 2a



Fig. 13.2b

et al 1966) The uterine short adrenergic neurons have rec ived particular attention because their transmitter content is influenced by female sex hormones probably from the ovary (see Falck et al 1972b) There was no seasonal variation in either the intensity or number of fluorescent cells and nerves in any of the guinea-pig tissues studied

Hedgehog The fluorescence microscopic pictur of the hedgehog pituitary was similar to that described for the guinea-pig although the number of faintly formaldehyde-fluorescent ells in the anterior lobe was smaller and the neurohypophysi contained a more scarc amount of catecholamine-containing nerve terminals

The adrenergi nerve supply to the thyroid gland losely resembled the situation i the guinea-pig. The calcitonin cells did not exhibit any formaldehyde-induced fluorescence I another hibernator the bat the thyroid gland has been found to contain C cells which store fluorogenic compound that has been interpreted as 5-HT (Gershon and Nunez 1970) Serotonin has also been demonstrated in calcitoni cells of few non-hibernators like sheep and goat (Falck et al 1964)

The adrenergic innervation of the heart in ertain hibernators including hedgehog has previously been studied i some detail (Ni lsen and Owman 1968) since preliminary observations (Nielsen and Owman 1965) had indicated a distribution of the cardiac nerves different from that i non-hibernators The present study has confirmed that i the hedgehog the cardiac adrenergic innervation is in general less pronounced than in the guinea-pig (Fig 13 1 com-

Fig 13 1 (a) Guinea-pig heart atrium spring Plexus of adrenergic nerve terminals which run both around vessel and i the myocardium proper Magnification indicator 100 µ. (b) Hedgehog heart atrium spring The amount and general distribution of fluorescent terminals esembl those of the guinea-pig atrium Magnification indicator 100 µ

Fig. 13 2 (a) Guinea-pig heart left ventricl spring The green-fluoresc nt adrenergi nerve terminals ar somewhat less numerous than i the atrium but their mod of distribution is the same to blood vessel and to the muscle fibres Magnification indicator 100 µ (b) Hedgehog heart left ventricle spring The sympatheti nerves are smaller i number than i the atrium and also less numerous than i the guinea-pig left ventricl However the innervation i different i that the terminals are found exclusively i relation to blood vessel Tiny autofluorescent (brown-y llow) pigment "dots are spread ll over the musculature Magnification indicator 100 µ



Fig. 13.3a

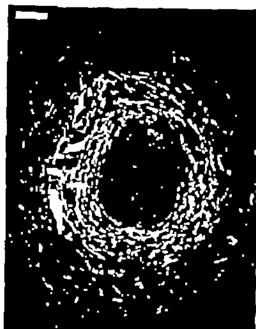


Fig 13.3b

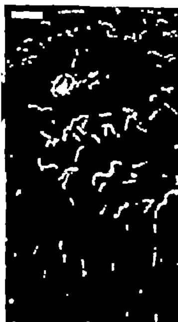


Fig. 13.4a

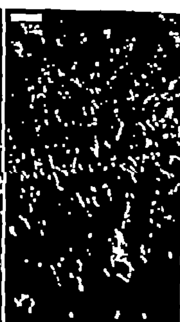


Fig 13.4b

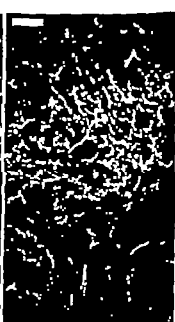


Fig 13.4c

pared with Fig 13 2) and that the number of nerves is larger in the atria (Fig. 13 1 b) than in the ventricles (Fig. 13 2 b) In the atria it seemed quite obvious that the nerves supplied both the vascular system and the musculature After visualizing the blood vessel in the heart by India ink infusion It has previously been found that the adrenergi nerves i the ventri les on the other hand exclusively accompany blood vessels thus leaving the myocardium proper without adrenergic innervation (Nielsen and Owman 1968) The total or almost tot l abs nc of muscular adrenergic nerves in the ventricles was confirmed also for ground squirr l (Citellus tridecemlineatus) and bat (Nyctalus noctula) (Nielsen and Owman 1968) Our interpr tation of the sults does not agree with that later presented by Angelakos et al (1971) on the basis of similar chemical and histochemical study on the ardiac adrenergic innervation of hamster ground squirrel and woodchuck. The latter authors use the similarity in the chemically determined cardiac concentration of norepinephrine between hibernators and non-hibernators to argue against a different distribution of ventri ular adrenergic nerves Further without having specifically demonstrated the vascular system for exampl through India ink infusion they claim that in the fluoresc nc microscope adrenergic fibres were egularly found among myocardial fibres bearing no lationship to the blood vessels

Adrenergic nerves were present in the vas deferens with the same distribution as in the guinea-pig but the density of the nerve network was lower in the hedgehog (Fig 13 3 b) This agrees with the lower oncentrations of norepinephrine eported by Sjöstrand (1965)

Fig 13 3 Transverse sections of vas deferens winter Magnification indicators: 100 μ (a) Guinea-pig Extremely dense adrenergi innervation of both the outer longitudinal (upper part of figure) and circular (middl part of figure) smooth muscl layers Rounded non-fluorescent area below t mucosa and lumen (b) Hedgehog Although the adrenergic innervation is less dense than i the guinea-pig vas deferens it i still very well developed particularly i the ircular muscl layer

Fig 13 4 Transverse sections of uterus The more peripheral part of the organ ircumference is i the upper portion of the figure mor centrally located parts (in direction towards the endometrium) i lower portion Magnification indicators: 100 μ (a) Guinea-pig summer Moderate number of green-fluorescent adrenergi nerve terminal i the myometrium (b) Hedgehog winter The adrenergic innervation i the myometrium i more pronounced than i the guinea-pig uterus ( ) Hedgehog summer The amount of adrenergi nerve terminal has increased markedly which is well illustrated by the very dense andrenergi nerve supply i the outer parts of the myometrium

The uterus was the only organ in the hedgehog showing a distinct seasonal variation in the adrenergi innervation As a general feature the network of varicose nerve terminals was better developed than in any hitherto investigated species (see Sjöberg 1967) excluding cat whi h has a nerve supply (Rosengren and Sjöberg 1967) that much resembles the pictu re found i the hedgehog (Fig 13 4 b and 13 4) The nerves in the outer longitudinal layer radiated i relatively small number from the surfac in direction towards the circular layer These fibres appeared to be mainly vasomotor A dense vascular supply was seen to those numerous vessels running between the two muscl layers. The circular layer had very high concentration of green-fluorescent nerve terminals which mainly ran in the direction of and lose to the smooth muscle fibres which they probably innervat A small number of isolated nerve fibres lso coursed into the mucosa some enclosing blood vessels other running immediately beneath the glandular epithelium During fall and winter the mucosa was relatively low In spring and summer the entire wall of the uterus had increased in thickness, particularly the mucosa whi h contained numerous tortous glands The number of nerves in the outer longitudinal layer had increased I the circular layer the adrenergic nerve plexus had the same density as during fall and winter In spite of the marked increase in volume of the smooth musculature Thi would mean that the total number of fluorescent nerves had increased during spring and summer (Fig 13 4 b and 13 4 c) Some of the animals even had a denser adrenergic nerve net i thei more voluminous uterus during spring and summer compared to the situation during fall and winter It is possible that the increased level of neuronal norepinephrine in the uterus of the hedgehog during its active period is natural counterpart to the higher content of uterine norepinephrine that can be induced by estrogen treatment of the rabbit (Sjöberg 1968b Falck et al 1969)

Adrenergic nerves were never found i the parenchyma of the brown adipose tissue, lthough a small number of blood vessels were accompanied by fluorescent fibres. It appears that parenchymal fluorescent nerve fibres i fat are very difficult to visualiz (Wirsén 1964) unless the tissue is subjected to special treatment for exampl incubation in buffer containing α-methylnorepinephrine (Wirsén and Hamberger 1967) It is thus not unlikely that norepinephrine (or its fluorophore) escapes from the nerves during the histotechnical procedure or that the concentration of norepinephrine lready in the labile fat tissue is beyond the level of its histochemical detection (cf Alm et al 1967) The possibility should also be considered that α-methyl-norepinephrine is take up and visualized i system of non-adrenergi nerves in the adipose tissue

Chapter 14

Ti sue Activato of Fibri olysis I the Hedgehog

by

Maurizio Pandolfi Anders Bjernstad and Hans Ericsson

Tissues contain a fibrinolytic enzyme tissue plasminogen activator (TPA) Evidence has accumulated that this agent has an antithrombotic function and is involved in tissue repai processes (Astrup 1968) TPA is not uniformly distributed i the tissues but its level seems to be in direct proportion with the vascularization There ar notabl exceptions such as the liver and the renal cortex which although highly vascularized have neglectabl fibrinolytic activity (Astrup 1966) The same tissues or organ can show marked variations of fibrinolytic activity during its development (Pandolfi 1967) or during different functional states (Albrechtsen 1959) This paper is concerned with the assay of TPA i the heart lung and kidney of hibernating and non-hibernating hedgehogs

Methods and materials

Hedgehogs were killed by exsanguination under general anesthesi and portions of their heart lungs and kidneys were removed frozen and stored i sealed plasti bags at 20° C as noted in the General Methods and Materi l section (Chapter 2) The fibrinolyti activity of these organs was assayed by modification of the histochemical method of Todd (1959) Briefly sections were cut at 8 μ on cryostat microtome collected on ordinary glass slides and covered with mixture of 0 06 ml 1% bovine fibrinogen in phosphate buffer pH 7 8 μ 0 15 prepared according to Brakman (1967) and 0 01 ml thrombin (20 NIH μ/ml unbuffered saline) The mixture was spread over an area of 10 sq cm. I order to obtai a fibri film 0 17 mm thick. Thereafter the film was llowed to stabilize at room temperature (21 24 C) i moist hamber Thereafter the lides were transferred into another moist hamber at 37° C Four slides were prepared for each organ and incubated for 15 30 45 and 60 minutes respectively After incubation the slides wer fixed in formalin and stained with Harris hematoxyli Fibrinolysis was eveded by colorless gaps i the fibrin film at the active cell of the section The strength of fibrinolysis was assessed on the basis of the incubation time required to produce lysis and the size of the lyti areas and i

expressed in arbitrary units as described by Pandolfi _t al_ (1967) Comparisons of the intensity of fibrinolyti activity was made by the rank-sum test of Wilcoxon

Results

All the organs of the animal sacrificed i the spring (III) showed fibrinolytic activity confined to small blood vessels (Fig 14 1 3) The localization remained unchanged in the organs from the nimal sacrificed in autumn (I) and during hibernation (II) The fibrinolytic strength of organs examined i summari ed in Table 14 1 I spring (III) lungs kidneys and to a lesser degree hearts show fairly high fibrinolytic activity I the autumn (I) and winter (II) the content of TPA decreased ignificantly i ll the organs (Lungs: spring (III) vs autumn (I) P 0 001; spring (III) vs winter (II) P 0 01 Hearts spring (III) vs autumn (I) and vs winter (II) P 0 01 Kidneys: spring (III) vs autumn (I) P 0 01; spring (III) vs winter (II) P 0 05) No ignificant differences were see between the activity of the organs during autumn (I) nd winter (II)

Discussion and conclusion

The esults show lear decrease of TPA i the lungs heart and kidney of hedgehog immediately before and during hibernation These organs have been demonstrated to be especially rich i TPA (Albrechtsen 1959) and they have been selected on the assumption that they would be mor sensitive t hanges of TPA The reason for thi decrease is obscure TPA is an enzyme connected with cell lar function more than with growth and reproduction and its decrease is i good agreement with the general abatment of ital functions occurring during hibernation.

Fig 14 1 P lymorphous areas of fibri digestion (lear zones) produced by section of lung Fibrinolysi originating from blood vessel The bronchi are inactive Incubation time: 30 minutes (x25)

Fig 14 2 Cross section of the heart passing through the ventri les Several punctat areas of lysis i the myocardium Discrete fibrinolysi produced by the endocardi l cells (arrow) Incubation time: 45 minutes (10)

Fig 14 3 Section of the kidney including cortex (left) and medull (right) Fibri digestion present at th medulla; the ortex i not active Incubation time: 30 minutes (x10)

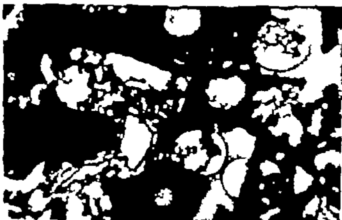

Fig 14.1


Fig 14 2


Fig. 14 3

Table 14 1

Fibrinolyti activity i arbitrary units of lungs (L) hearts (H) and kidneys (K) of hedgehog in spring (III) autumn (I) and winter (II)

Spring III			Autumn I			Winter II		
L	H	K	L	H	K	L	H	K
8			0	0	1	2	0	0
0	1	7	4	0	3	0	0	4
6	1	7	1	0	2	4	0	5
8	2	7	1	0	0	2	0	2
9	2	9	2	0	2	1	0	4
12	6	6	1	0	3	0	0	4
19	4	4	3	0	4	2	0	
			2	0	1	3	0	4
			2	0	6			
			1	0	4			

On the other hand owing to the assumed anti-thromboti function of TPA (Nilsson and Pandolfi 1970) one would expect an increase of the enzyme i time when the risk of thrombosis is higher because of the lower irculation speed However many factors are involved i the etiology of thrombosi and it i possible that other compensatory mechanisms are activated i the hibernating animal i order to maintai thei vascular patency (Biörck et al 1962)

Chapter 15

Ult st uctu e f Brow Adipose Tis ue and Myoc dium

by

Sven-Oll R. Olsson

The following questions are of interest i this seasonal study of hedgehogs and gui ea-pigs

1 Are there any seasonal changes in the ultrastructur of brown adipose tissue of hedgehogs ? Reviews hav been published by Rasmussen (1923) Johansson (1959) and Lindberg (1970)

2 Ar there any ultrastructural differences betwee the myocardium of hedgehogs and guinea-pigs? Such differences could help to explain the differences i temperature tolerance between the myocardium of hibernators and non-hibernators (Johansson 1967 1969 a and b and Chapter 11)

3 Are there any adaptive ultrastructural differences i the myocardium between hiber nating or non-hibernating hedgehogs? Such differences have been reported from the ground squirrel (Citellus tridecemlineatus) by Rosenquist and Zinny (1969) and Rosenquist (1970) but opposing results have been found from the ground squirrel (Citellus lateralis) by Aloi and Pengelley (1971)

Method

The apex of the left ventri l was excised from four hibernating (II) and four non-hiberna-ting (IV) hedgehogs and from four guinea-pigs during winter (II) and four during ummer (IV) (See General Methods and Materials Chapter 2 Fig 2 1) The piec of myocardium was ut into 1 mm cubes. From the same hedgehogs pieces of brown adipose tissue wer excised and lso ut into 1 mm cubes

The pieces of tissue were fixed i 3% glutaraldehyde i phosphate buffer (Millonig 1961) washed in buffer postfixed i 1% osmium tetroxide i phosphate buffer (Millonig 1961) de-dehydrated i thanol block st ined i 1/2% uranyl acetate and 1% phosphotungsti acid i ethanol and embedded i Vestopal W® (Marti Jaeger Geneva Switzerland) The em-bedded tissues were sectioned and then stained with 1% uranyl acetat and 0 1 g/100 ml

lead itrate[1] The sections were studied with a Philips EM 300 and Hitachi HS-75

Results

Brown adipose tissue No differences in the shape of size of nuclei were found from hibernating and non-hibernati g hedgehogs The si of the nucleus i 4-6 μ

A greater number of mitochondria per adipocyt were observed in brown fat from hibernating hedgehogs (Fig 15 1 b) as compared to non-hibernating (Fig 15 1 a)

The shape of the mitochondria i polygonal from hibernating and lmost circular from non-hibernating hedgehog The arrangement of crista from mitochondria from both seasons is long lender gently urved and non-branching cristae eachi g from one side of the mitochondrion to the other

Ther are no significant differences of the izes of mitochondria (1 2 μ) from hibernating or non-hibernati g hedgehogs

The number of fat droplets per adipocyt was greater i tissue from hibernating hedgehog than from non-hibernating However the fat droplets were larger from non-hibernating (5-10 μ) compared to hibernating hedgehogs (1 5 μ) It is quite obvious that the amount of fat per adipocyt i significantly greater from the non-hibernating hedgehog compared to the hibernating hedgehog (Fig 15 1)

The shape of the fat droplets is always spheric l

---

1 a) 1% uranyl acetate i 75% ethanol 20 min at 20° C
 b) Washed i 50% thanol and aq dest and dried
) Lead itrat 0 1 g/100 ml 0 1N NaOH 2 min at 20° C
 d) Washed i distilled water

Fig 15 1 a Brown adipose tissue from summer hedgehog (IV) Big fat droplets (F) occupy most of the ll volume The umber of mitochondria (M) i sm ll Magnification 6 000 x

Fig 15 1 b Brown adipose tissue from hibernating hedgehog (II) The fat droplets (F) are smaller but mor numerous and the number of mitochondri (M) i greater than from non-hibernating hedgehog Magnification 6 000 x.

Fig 15 1 c Brown adipose tissue from hibernating hedgehog (II) showing pinocytosis (P) Magnification 19 000

Fig 15 1 d Brown adipose tissue from summer hedgehog (IV) showing pinocytosis (P) Magnification 19 000

Fig 15 1 Brown adipose tissue from hibernating hedgehog (II) showing a developing brown adipocyt with an obvious sarcoplasti reticulum (SR) Magnification 19 000 x The line below the micrographs epresents 1 μm



Fig. 15.1a 1µm.



Fig. 15.1b 1µm.



Fig. 15.1c 1 µm

P

Fig. 15.1d 1 µm



Fig. 15.1e 1 µm

The sarcolemma has rather smooth structur lthough pinocytosis has been observed both from hibernating (Fig 15 1) and non-hibernating (Fig 15 1 d) hedgehog

The sarcoplasmi reticulum an hardly be seen from hibernating hedgehog but is more prominent from the non-hibernating hedgehog A well developed sarcoplasmic reticulum has been found i a developing adipocyte (Fig 15 1) from hibernating hedgehog.

Heart There were no apparent differences in the structure of the nuclei from myocardium of hibernating and non-hibernating hedgehogs For both seasonal stages the contours of the nucl i wer rather smooth (Fig 15 2 a) with distinct double layed nuclear membrane and with osmiophilic material concentrated to the periphery However the nucl i of the myocardium from guinea-pig were more irregular (Fig 15 2) as compared with the hedgehog In some nucl i nucleolus was visible

No obvious differences i the number of mitochondria from hibernating and non-hibernating hedgehog and guinea-pig have been found

The shape of the mitochondri are either ircular or elliptical depending on the direction of section No typi l long lender mitochondria have been found The ristae are usually long thi folds of the inner mitochondrial membrane reaching from one side of the mitochondrion to the other N branching of the crista has been observed There are no differences i the shape or number of cristae in the mitochondri from hibernating (Fig 15 3 b) or non-hibernating hedgehog (Fig 15 3 a) or guinea-pig (Fig 15 3 d) The number of cristae per mitochondrion is 20-30 Fig 15 3 b i an exception with about 50 ristae The thickness of the cristae varies between 10-20 mμ no signifi ant differenc between the species or seasons an be observed Some speci l mitochondria have been observed from hibernating hedgehog (Fig 15 3) and a guinea-pig (Fig 15 2) Thes giant mitochondri (Fig 15 3 c)

Fig 15 2 Heart muscl ll from hibernating hedgehog (II) showing part of typical nucleus (N) with nucleolus (NL) and some pinocytosi (P) Fl = muscl fiber Magnification 19 000

Fig. 15 2 b Heart muscle ell from summer hedgehog (IV) showing the typical zig-zac manner of the sarcolemma (S) at each Z line of the muscl fiber (Fl) Magnification 5 000

Fig 15 2 c Heart muscl ell from guinea-pig (IV) showing typical nucleus (N) the ig- ao manner of the sarcolemma (S) the muscl fibers (Fl) and strange form of mitochondria (M) with circular concentri crista Magnification 6 000 x

The line below the micrographs represents 1 μm



Fig. 15.2a



Fig. 15.2b
1μm



Fig. 15.2c
1μm

from one hibernating hedgehog are about 3–4 μ with a large number of densely packed cristae The cristae seems to be littl curved and non-branching and seems to cross the whol width of the mitochondrion These giant mitochondri ar not typical for hibernating hedgehogs as they have been found in only one hedgehog and not in guinea-pigs The special mitochondria from the guinea-pig (Fig 15 2 c) are of normal si but the cristae are arranged in lrcular manner

No significant difference in the si e of mitochondri between the species or seasons can be observed The ize of the mitochondria varies between 0.5–2 μ

The Intercalated discs are well developed i the myocardium of hedgehog and guinea-pig The continuity betwee sarcolemma and the intercalated discs i illustrated i Fig 15 4 a and 15 4 b The different structures of the intercalated disc (Dewey 1969) are seen in the direc tion of the muscl fibers in Fig 15 4 and in a direction perpendicular to the muscle fibers in Fig 15 4 b All these three components of the intercalated disc have normal mammalian appearanc There are no differences between guinea-pig and hibernating and non-hibernating hedgehog The interc ll lar space i the disc is about 2 mμ

The sarcolemma has a very special configuration i myocardium At each Z line of the myofibrills there is an indentation of the sarcolemma to make the distance between the sarcolemma and the Z line of the myofibrill as short as possibl Thi is illustrated in Fig 15 2 b and c It i evident from thi study that the frequency of pinocytosis is ather great There are no differences between guinea-pig hibernating or non-hibernating hedgehog in these respects

The two components of the sarcoplasmic eticulum are about equally developed i the two species and no differences between hibernating and non-hibernating hedgehog have been found. The transverse system (TS) is easily seen lose to the Z lines in Fig 15 4 b 15 5 and 15.5 b The longitudinal system can be seen in Fig 15 4 b and 15 5 a The internal oupling

---

Fig 15 3 a. A typi al mitochondri from summer hedgehog (IV) heart Magnification 32 000 x.

Fig 15 3 b A mitochondria with an unusual great number of cristae from hibernating hedgehog heart Magnification 32 000 x

Fig 15 3 c Heart muscle ll from hibernating hedgehog (II) with enormous great mitochondria with large number of cristae Magnification 6 600

Fig 15 3 d A typical mitochondri from guinea-pig heart Magnification 32 000 x.

The line below the micrographs represents 1 μm



Fig. 15.3a 1 µm



Fig. 15.3b 1 µm



Fig. 15.3c 1µm



1 µm

Fig. 15.3d

Fig. 15.4a

1 µm

Fig. 15.4b

1 µm

1 µm

Fig 15.5a



1 µm

Fig. 15.5b

curved and transverse the whole width of the mitochondria is quite in agreement with those of other mammals (Afzelius 1970) The multilocularity which is one of the characteristics of brown adipocytes is most typical during the hibernation of hedgehog The lipid droplets increase in siz from 1 5 µ to 5-10 µ from winter to summer The small size of fat droplets during hibernation favors the convenience of access of fat for catabolism This implies that the lipid content per adipocyte is greater during summer than during hibernation which I supported by the work of Paleus and Johansson (1968) who found maximum concentration of lipids in hedgehog brown fat during May-September The same esults have bee found by Suomalainen and Herlevi (1950) who found the most intensely sudan black stained brown adipocytes from summer hedgehogs and by Zirm (1956) who found a higher lipid content in brown fat from non-hibernating hedgehog than from hibernating It is obvious from these results that the synthesis of lipids starts lmost immediately after the arousal of hedgehog as has been pointed out by Boerner-Patzelt (1958) This is also supported by the increase in activity of glucose-6-phosphate dehydrogenase (G-6-PDH) between winter (II) and spring (III) in brown fat of hedgehog (Chapter 11) I the bat (Rhinolophus ferrum-equinum nippon) however Umahara (1968) revealed strong depletion of fat droplets from brown adipocytes in April and conspicuous atrophy of brown adipocytes in August This change was correlated to possible resting stat I summer It is evident that there are great species differences in the seasonal changes of brown adipocytes and that it is impossible to draw any conclusions from the results from one speci to another

The brown adipose tissue of hedgehogs from the spring season (III) (see Fig 2 1) has the same ultrastructural appearanc as from the hibernating season (II) (Fig 15 6)

This i quite i agreement with the fact that thes animals were almost hibernating (Chapter 2) Consequently the brown adipose tissue from these spring hedgehogs is neither disintegrated nor depl ted of fat (see Paleus and Johansson 1968) after all the arousals during the hibernation which implies that thes animals have some reserve apacity

Afzelius (1970) has eviewed the anatomy and cytology of brown adipose tissue and characterized the plasma membrane by its numerous invaginations Hedgehog brown adipose tissue has not been well investigated with egard to these invaginations which are generally considered to be an expression of pinocytoti activity

Fig. 15 6a



Fig. 15 6b 1µm

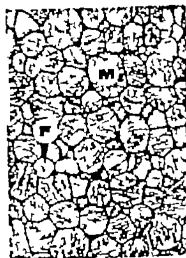


Fig. 15.6c 1µm

No differences in the structure of myocardium from guinea-pig hibernating or non-hibernating hedgehog were observed. The absenc of seasonal changes of the number of mitochondri in myocardium is supported by the result that mitochondrial MDH does not show any difference in percental activity from hibernating or non-hibernating hedgehog (Chapter 11). Poche (1959) found in the myocardium of hibernating fat dormouse (Gli glis) that the number of mitochondria was constant. The number of cristae and the contrast of the mitochondria increased however in comparison with the non-hibernarory state. On the other hand Moreland (1962) found an increase in the number and siz of mitochondria from the myocardium of the ground squirrel (Cit. tellus tridecemlineatus) when hibernating compared to non-hibernating. Zimny and Moreland (1968) confirmed these results on the same specie. During arousals the number of mitochondria decreased and the succinic dehydrogenase activity increased. Zimny and Moreland (1968) proposed that the increase of the number of mitochondria depends on increased fatty acid oxidation during hibernation which i supported by the proposal that the turnover of lipids i myocardium is increased during the hibernation of hedgehog (Chapter 11). The low value of the activity of succinic dehydrogenase during hibernation of ground squirr l (Citellus tridecemlineatus) may be an expression of low aerobic metabolism in the myocardium of hibernating animals. This is supported by the high anaerobic apacity of hibernators (Chapter 11).
Rosenquist and Zimny (1969) observed differences i the myocardi l ltrastructure between hibernating and non-hibernating ground squirrels (Citellus tridecemlineatus). The sarcoplasmic reticulum was more abundant during hibernation and the terminal isternae or lateral sacs of the myocardial tried wer mor electron dense than those not hibernating. I addition the transverse tubules near the ell periphery wer greater in diameter i the myocardium of hibernating ground squirrels. They lso observed that the cell coat thickness was thick (350 Å) during hibernation but only 250 Å i the non-hibernating rat. These results were later confirmed by Rosenquist (1970) who suggested that these ultrastructural hanges may enhance calcium upt ke and thereby facilitate excitation contraction oupling during hibernation.

Fig. 15.6 a A typical brown adipocyt from spring hedgehog (III). It i the same appearance as from hibernating hedgehog. Magnification 9 600

Fig 15 6 b A part of brown adipocyte from spring hedgehog (III) with many fat droplets (F). Magnification 6 000 x.

Fig 15 6 A part of brown adipocyt from spring hedgehog (III) with very few and small fat droplets (F). Magnification 6 000

The line below the micrographs represents 1 µm

Zimny et al (1968) observed that the intermembrane left I the intercalated discs were widened at 15° C-body temperatur of rats and that the width of the intermembrane cleft was maintained and that the intracytoplasmati density was increased I ground squirrels (Citellus tridecemlineatus) at body temperatures of 5° C compared to normal animal This effect in rats is difficult to interpret in physiological terms as Barr t al (1965) have shown that the desmosomal junction is a path of low resistance for the action potentials The action and the membrane potentials of rabbit atria have been shown to diminish rapidly between 25-15° C but this is not the ase for ground squirrels (Citellus tridecemlineatus) between 31-6° C body temperature (Marshall and Willis 1962) Consequently there are also great differences I the myocardial membranes from hibernators and non-hibernators besides the biochemical differences (Chapter 11)

The changes of the myocardial ltrastructur reported by Rosenquist and Zimny (1969) and Rosenquist (1970) in the myocardium of the ground squirrel (Citellus tridecemlineatus) can neither be supported by this investigation on hedgehogs nor by that of Aloi and Pengelley (1971) on Citellus lateralis, which I a close relative to Citellus tridecemlineatus Aloi and Pengelley (1971) did not find any ultrastructural differences in the myocardium of hibernating and non-hibernating ground squirrel or between the ground squirrel and other mammalian species which is quite I accordanc to the esults of this study on hedgehogs and guinea-pigs

Chapter 16

G n al S mmary

1 A study of the seasonal variations of various physiological and biochemical variables was performed on the European hedgehog (Erinaceus europaeus) the guinea-pig (Cavia porcellus) and man (Homo sapiens)

2 Samples were taken at four periods during the year (designated I–IV); these periods corresponded to f ll winter spring and summer

3 The variables measured were the following: body weight sex temperature electrocardiogram blood PaO_2 $PaCO_2$, pH and standard bi arbonat blood glucose and hemoglobi Also measured wer serum concentrations of sodium potassium lcium hloride urea creatinine total protein insulin lactic dehydrogenase total fat triglycerides free fatty acids and cholesterol Tissue samples were taken from the animal subjects for monoamine oxidase (MAO) catecholamine-O-methyl transferase (COMT) and phenyl ethanolamine-N-methyl transferase (PNMT) from heart and adrenal Epinephrine and norepinephrine concentration i heart and adrenal lactic dehydrogenase (LDH) mal dehydrogenase (MDH) alpha-glycerol phosphate dehydrogenase (GPDH) and glucose-6-phosphate dehydrogenase (G-6-PDH) activity in heart liver brown fat and white fat were also measured Tissue samples were also taken from heart kidney and lung for fibrinolytic activity white and brown fat for fatty acid composition of tissue lipids from heart and liver for glycogen concentration and from heart and brown fat for ultrastructure The weight of heart adrenals liver pancreas spleen testes ovaries and brown fat was also recorded

4 The body temperature of the hedgehog was lowest during the hibernating season II) with an average of about 10 C The guinea-pig showed constant body temperature in all seasons.

5 The hedgehog has mi imum body weight during the arousal season (III) and maximum weight in the F ll (I) The guinea-pig i heaviest i the winter (II) and lightest i the summer (IV)

6 Th heart in both species is heaviest i winter (II) and lightest i summer (IV)

7 Liver weight seems to parallel body weight in both species

8 The spleen weight parall ls body weight i the hedgehog and does not hange in the guinea-pig

9 Brown fat weight i highest in f ll (I) and decreases to mi imum in summer (IV) i the hedgehog No samples were obtained from the guinea-pig

10 Testes weight i the hedgehog was highest i the summer (IV) and lowest i the fall (I) The data on uterine and ovarian weight was not significant i either species.

11 The electrocardiograms showed lack of S-T segment i the hedgehog The heart rates were lowest in the hedgehog in the hibernating season (II) (13 7 beats per minute) and highest i season III The guinea-pig showed lowest heart rat i summer (IV) and highest in winter (II) I man the highest heart rat was seen i fall (I) and lowest i summer (IV)

12 Serum sodium was highest in summer (IV) and lowest in spring (III) in the hedgehog In the guinea-pig serum sodium was highest in summer (IV) and lowest in winter (II) and spring (III) Serum chlorid values f llowed hanges in sodium

13 Serum potassium is high i f ll (I) and low in summer (IV) i man i the hedgehog serum potassium is lowest i the winter (II) whil i the guinea-pig the serum potassium is highest i winter (II)

14 I man serum calcium was lowest i fall (I) The serum cal ium i the hedgehog was highest in winter (II) The guinea-pig showed changes in serum calcium similar to those seen in the hedgehog

15 The hedgehog showed the highest PaO_2 values in winter (II) whil the guinea-pig showed higher values in summer (IV) and lowest i f ll (I) The $PaCO_2$ values i hedgehogs were highest i winter (II) No seasonal hanges were seen in [illegible] the $PaCO_2$ values

for all seasons wer higher in hedgehogs than guinea-pigs. The blood pH vol es lowest in winter (II) for the hedgehogs The guinea-pig showed higher pH values I winter (II) and lowest in summer (IV) The guinea-pig showed higher pH values for all seasons than did the hedgehog.

16 Serum protein values were lowest I guinea-pigs and higher I man and hedgehogs In man the highest serum protein occurs in fall (I) Serum protein was lowest I spring (III) i both guinea-pigs and hedgehogs The serum urea values wer highest I hedgehogs and lowest in man In the hedgehog lowest values were seen I fall (I) and in the guinea-pig the highest values wer seen I summer (IV)

17 Blood glucose was lowest in winter (II) in the hedgehog Slight decreases were seen in all three species I ummer (IV) Pancreatic insuli was lower in fall (I) and winter (II) than in summer (IV) in the hedgehog and circulating insuli could not be measured

18 Plasma lipids showed lower values during summer (IV) for man and hedgehog and lower values I spring (III) for guinea-pig. The major changes were seen in cholesterol with man and hedgehog showing highest values I winter (II) The guinea-pig had cholesterol values about 25% of those seen in man and hedgehogs Free fatty acids were higher I wi ter (II) in the hedgehog lipoprotei patterns did not show seasonal variation

19 Gas chromatography revealed that there were more long chain fatty acids I brown fat than white fat in the hedgehog and lowest I whit fat from guinea-pigs The degree of unsaturation was higher I brown fat than white fat I the hedgehog with no seasonal change in saturation Guinea-pig whit fat showed total unsaturation about one-half that seen I the hedgehog except during summer (IV) when linolei and linolenic acid increased I the whit fat from the guinea-pig Linol I acid increased I both animals in the summer season (IV)

20 The seasonal changes of th activities of the four dehydrogenases LDH MDH G-6-PDH and α-GPDH from heart liver white and brown fat of hedgehog have one common feature In all organs except white fat ll enzymes have maximal activity during hibernation G-6-PDH in brown fat and α-GPDH I brown fat and liver are exceptions Seasonal changes in activities of these four enzymes from the same organs of guinea-pig are quite different from that of hedgehog. Only α-GPDH from hedgehog and guinea-pig heart show similar changes. The activities of LDH and α-GPDH I heart and G-6-PDH I liver from hedgehog are significantly higher than the corresponding activities from guinea-pig On the other hand

activities of MDH and G-6-PDH i heart α-GPDH in liver and MDH G-6-PDH and α-GPDH in white fat from guinea-pig are significantly higher than th activities from hedgehog These results may indicat the following differences between the hedgehog and guinea-pig:

1 higher anaerobic capacity for heart and liver of hedgehog than guinea-pig.

2 higher capacity for the pentose-shunt i hedgehog liver and i guinea-pig heart and white fat than other tissues examined (see discussion in the section on G-6-PDH)

3 higher capacity for glycerol metabolism i hedgehog heart and i liver and white fat of guinea-pig than i other tissues examined

21 The concentration of norepinephrine (NE) in the hedgehog heart's left ventri l was about one-third that of the guinea-pig The seasonal variation showed a minimum i fall (I) to maximum in summer (IV) The guinea-pig showed littl change except an increased heart NE level in summer (IV) Monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) wer highest i spring (III) and lowest i summer (IV) and fall (I) i the hedgehog heart MAO was maximal i spring (III) and COMT i winter (II) i the guinea-pig heart Phenylethanolamine-N-methyltransferase (PNMT) was low i hearts of both species and seasonal variations were lacking.

22 I the adrenal glands of both species the concentration of epinephrine (E) was much higher than NE The E/NE ratio was lower in the hedgehog than the guinea-pig. The E/NE rati i the hedgehog adrenal was maximal i winter (II) I the hedgehog E concentration was constant throughout the year whil NE was high i f ll (I) and summer (IV) and low in winter (II) and spring (III) I the guinea-pig adrenal NE increased from fall (I) steadily to maximum i summer (IV) while E followed the same pattern seen for NE i the hedgehog adrenal MAO and COMT were highest i winter (II) i the guinea-pig adrenal and lowest in f ll (I) PNMT was maximal i winter (II) and minimal i summer (IV) in the guinea-pig adrenal MAO and COMT activities wer highest i winter (II) and spring (III) and lowest in fall (I) and summer (IV) i hedgehog adrenal The PNMT activity decreased from higher levels during fall (I) to low level i summer (IV)

23 Fluorescenc microscopy of pituitary thyroid ight atrium left ventricl vas deferens and uterus from guinea-pig and the same tissues together with interscapular brown fat in hedgehog yi lded only one seasonal variation The uterus i the hedgehog showed more adrenergi nerve fibers i spring and summer This corresponds to the presumed seasonal reproductive activity illustrated by the organ weights

24. Tissue fibrinolytic activity was lowest in autumn (I) i heart lung and kidney f om hedgehogs The spring (III) and winter (II) values were similar There was maximum acti ity i all tissues in the summer season (IV)

25. Electron microscopy showed no seasonal changes in ultrastructure of the heart of hedgehogs and guinea-pigs and no structural difference between the two I brown fat a higher number of mitochondria were seen in hibernating (II) as opposed to non-hibernating (IV) hedgehogs The size of fat droplets in the brown fat is also decreased in the hedgehog during hibernation

26. The above data interpreted broadly support the existence of three different physiological responses to seasonal changes among the three species examined First the hedgehog hows metabolic and physiological adjustment preparing for during and after the depressed metabolic state of hibernation. Second the guinea-pig shows much less seasonal hange the seasonal changes seen are directed at increased metabolic heat production during the winter season Third man shows even less seasonal change probably due to less actual exposure to the limatic effects of season. I addition to temperature effects the metaboli hanges seen in the three species may lso be related to photoperiodi dietary or seasonal breeding influences.

Appendix table

Sex	Age	Hemoglobin mg % I	II	III	IV	Blood glucose mg % I	II	III	IV	Chloride mEq/l I	II	III	IV	Serum protein g % I	II	III	IV
♀	32	12.8	13.9	12.8	13.1	80	77	54	70	114	105	106	106	—	7.8	7.3	7.4
♀	21	13.3	12.8	13.3	12.8	67	76	75	70	112	106	107	106	7.9	7.5	7.7	7.4
♀	40	13.5	14.4	13.3	14.4	91	94	80	67	110	106	107	106	7.7	7.9	8.1	7.9
♀	24	14.3	13.9	14.3	13.1	73	74	103	67	110	107	105	—	7.8	8.0	7.5	7.3
♀	22	13.1	12.8	12.8	12.6	69	74	75	73	111	107	109	—	—	7.9	7.7	7.4
♀	26	13.3	14.3	13.7	13.3	67	76	80	68	110	106	107	113	7.6	7.7	7.8	7.5
♂	37	14.3	14.3	13.9	14.8	84	91	81	75	110	104	106	—	8.0	7.4	7.1	7.8
♂	29	14.8	15.4	14.6	14.1	90	79	65	68	106	101	97	—	8.0	7.8	7.4	7.3
♂	34	15.2	14.3	15.6	14.6	92	73	92	74	112	107	108	—	8.0	7.6	7.5	7.3
♂	30	14.6	14.8	15.0	14.4	63	62	87	77	103	112	116	106	8.0	8.1	—	7.4
[illegible]	[illegible]	15.4	14.6	16.6	15.2	79	84	84	66	109	107	110	106	8.6	7.8	7.8	8.1
[illegible]	[illegible]	15.9	16.3	15.4	15.6	89	80	77	72	109	107	—	106	7.7	7.4	8.9	7.2
[illegible]	[illegible]	15.2	16.9	16.7	16.5	102	87	86	56	106	105	102	106	7.7	7.5	7.9	7.6
Mean		14.3	14.5	14.4	14.2	82	79	81	71	110	106	107	107	7.9	7.7	7.6	7.5
SD		1.00	1.17	1.22	1.18	10.9	8.6	11.2	7.0	2.6	2.5	4.5	2.6	0.27	0.23	0.35	0.27
SE		0.28	0.33	0.34	0.33	3.0	2.3	3.1	2.0	0.7	0.7	1.3	0.9	0.08	0.06	0.10	0.07
n		13	13	13	13	13	13	13	13	13	13	12	8	11	13	12	13

APPENDIX.

Individual values, standard deviation (SD), standard error (SE) and number of observations (n) in human subjects during the four seasons. Roman numbers refer to season. See further chapter 9, Fig 1

Appendix table (cont.)

Sex	Age	Urea mg %				Sodium mEq/l				Potassium mEq/l				Calcium mEq/l			
		I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
♀	32	36	36	40	23	146	140	140	144	4.8	4.7	5.0	4.5	5.3	5.4	5.1	5.6
♀	21	27	36	25	26	148	140	144	143	4.9	4.4	4.6	4.6	5.4	5.3	5.4	5.6
♀	40	22	32	33	27	146	140	143	142	4.4	4.3	4.6	4.0	5.3	5.4	5.1	5.7
♀	24	26	23	27	21	147	142	138	143	5.0	4.4	4.4	4.3	5.5	5.6	5.3	5.4
♀	22	28	21	18	22	145	142	147	142	4.8	4.4	4.8	4.5	5.3	5.4	5.3	5.2
♀	26	23	19	24	25	145	142	142	135	4.6	4.4	4.2	3.6	5.3	5.3	5.4	5.2
♂	37	33	23	22	21	146	138	142	143	5.1	4.3	4.3	4.3	5.4	5.4	5.3	5.4
♂	29	36	28	26	25	145	140	144	142	4.9	4.4	4.5	4.6	5.4	5.5	5.4	5.3
♂	34	45	47	30	34	146	150	143	143	4.8	4.6	4.3	4.5	5.3	5.3	5.3	5.2
♂	30	31	45	27	26	142	152	153	145	4.7	4.3	4.4	4.0	5.0	5.7	5.5	5.6
♂	35	25	31	28	31	146	145	144	143	5.3	4.8	5.0	4.8	5.4	5.8	5.7	5.6
♂	28	32	26	38	30	143	140	143	139	4.8	4.6	4.8	3.9	5.2	5.8	5.1	5.7
♂	38	50	50	43	47	145	146	140	143	4.4	4.6	4.3	4.4	5.1	5.1	5.6	5.4
Mean		32	32	30	28	145	143	143	142	4.8	4.5	4.6	4.3	5.3	5.5	5.4	5.5
SD		8.4	10.3	8.1	7.0	1.4	4.2	3.7	2.5	0.26	0.16	0.27	0.30	0.14	0.21	0.19	0.19
SE		2.3	2.9	2.2	2.0	0.4	1.2	1.0	0.7	0.07	0.04	0.08	0.08	0.04	0.06	0.05	0.05
n		13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13

Appendix table (cont.)

Sex	Age	Creatinine mg %				LDH				GOT				GPT			
		I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
♀	32	—	0.8	1.25	0.8	310	235	270	305	—	17	32	29	—	14	28	20
♀	21	0.85	0.85	0.8	0.7	270	270	285	315	16	22	19	16	17	22	10	14
♀	49	1.0	1.05	0.75	1.0	350	380	350	425	16	17	13	12	17	24	14	13
♀	24	0.95	0.85	0.9	0.96	320	380	350	390	14	16	23	15	10	14	11	14
♀	22	—	0.8	0.9	0.8	310	320	310	365	17	16	23	16	16	17	16	21
♀	28	0.8	0.8	0.95	0.85	210	240	250	240	13	14	12	14	12	15	10	14
♂	37	1.15	1.2	1.1	1.1	320	290	280	300	22	20	19	17	20	24	14	18
♂	29	1.2	1.1	1.1	1.05	450	460	410	600	18	19	18	19	17	16	13	17
♂	34	1.05	1.05	0.95	0.9	350	320	390	385	16	4	17	18	12	7	11	18
♂	30	1.1	1.1	—	1.0	380	380	260	325	22	21	24	24	20	22	24	14
♂	35	1.2	1.26	1.36	1.1	290	270	330	375	12	17	17	27	15	20	14	23
♂	28	1.0	0.85	1.0	1.06	410	440	370	405	24	27	22	23	25	31	19	25
♂	38	1.05	1.1	1.0	1.2	410	245	560	385	19	—	27	28	14	—	19	19
Mean		1.03	0.96	1.00	0.96	335	322	338	363	17	18	20	20	16	19	16	18
SD		0.13	0.16	0.17	0.14	64.0	72.6	86.0	66.1	3.8	5.5	6.6	5.6	4.1	6.2	5.2	3.8
SE		0.04	0.06	0.05	0.04	17.8	20.1	23.6	18.3	1.1	1.6	1.5	1.6	1.2	1.8	1.4	1.1
n		11	13	12	13	13	13	13	13	12	12	13	13	12	12	13	13

Appendix table (cont.)

Sex	Age	Total fat mg %				Triglycerides mM				Free fatty acids mM				Cholesterol mg %			
		I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
♀	32	—	742	791	737	0.73	0.45	0.58	0.92	0.59	0.35	0.48	0.52	180	227	195	100
♀	21	678	538	864	899	0.61	0.67	0.89	0.64	0.61	0.36	0.44	0.34	174	159	155	100
♀	49	735	804	889	722	0.96	1.22	1.14	1.1	0.56	0.68	0.30	0.63	163	193	180	165
♀	24	864	704	681	599	0.49	0.48	0.64	0.46	0.28	0.55	0.55	0.77	161	195	195	175
♀	22	863	704	858	571	0.28	0.34	0.56	0.27	0.87	0.58	0.50	0.63	180	192	175	160
♀	26	658	588	643	492	0.52	0.52	0.49	0.62	0.42	0.58	0.38	0.43	138	154	155	135
♂	37	786	699	891	717	0.48	0.51	0.53	0.68	0.38	0.28	0.37	0.54	203	218	180	185
♂	29	870	745	858	899	2.05	1.11	1.80	1.7	0.42	0.44	0.60	0.61	185	191	175	180
♂	34	571	489	481	471	0.50	0.78	0.62	0.74	0.41	0.15	0.22	0.15	144	148	140	135
♂	30	899	719	517	512	0.64	0.58	0.31	0.33	0.53	0.49	0.12	0.24	156	184	150	130
♂	35	909	947	799	804	0.82	0.64	0.51	0.55	0.36	—	0.22	0.56	204	288	220	240
♂	28	799	820	855	889	0.76	0.78	0.80	0.88	0.29	0.44	0.37	0.92	218	253	200	210
♂	38	712	776	889	724	1.06	0.92	0.15	0.95	0.31	0.41	0.68	0.35	207	225	205	185
Mean		719	712	880	641	0.76	0.69	0.66	0.74	0.48	0.44	0.40	0.51	178	201	177	162
SD		106.1	123.5	122.7	106.8	0.44	0.26	0.41	0.37	0.17	0.14	0.16	0.21	25.1	36.6	24.5	41.0
SE		30.6	34.3	34.0	29.6	0.12	0.07	0.11	0.10	0.05	0.04	0.04	0.06	7.0	10.[illegible]	6.8	11.4
n		12	13	13	13	13	13	13	13	13	12	13	13	13	13	13	13

Acknowledgement

Portions of this work were supported by an NIH postdoctoral fellowship (1-F2 24 990-01) to Jerome B Senturi Computer time was supplied by the Cleveland Stat University Miss J Szilagyi prepared most of the illustrations Miss B Eklund Miss C Nyquist and Mrs A -C Johansson provided technical and secreterial assistance throughout the study Mrs M. B Nilsvik aided in the study of fatty acid composition (Chapter 10) and Mrs B Carlsson and A -G Petersen in the study of th enzymatic activity (Chapter 11) and ultrastructure (Chapter 15) V luable printing advice was provided by Mr A Andersson The assistance of the head and personal of the Department of Clinical Chemistry and Department of Cli ical Physiology is appreciated

The work in Chapter 8 was supported in part by the Swedish Medical Research Council (grant No B72-13 -3137-02) and in Chapters 12 and 13 i part by the Association for the Aid of Crippled Children and by the Ford Foundation (grant No 68-383) New York.

We gratefully acknowledge the encouragement and support of Dr Frank DeMarinis and Dr John H Morrison during their respective tenure as chairman of the Department of Biology at the Cleveland State University

Valuable and generous help was given by AB Ferrosan Malmö

Further support to the study was given by Faculty of Medicine of the University of Lund Gunborg Lundéns donation Faculty of Medicine Hulda Almroths stiftelse Nordisk Insulinfond Swedish National Association against Heart and Chest Diseases The Royal Physiographical Society of Lund and Victor and Albertina Mollinders fond Swedish Medical Society

References

ADROUNY G A. Differenti l patterns of glycogen metabolism in cardiac and skeletal muscles. Amer J Physiol 1969 217 686

AFZELIUS B A Brown adipose tissue: it's gross anatomy histology and cytology I Brown Adipose Tissue (Edited by Lindberg O) Elsevier 1970

AGID R. and L. AMBID Effects of corporeal temperature on glucose metabolism in a homeotherm the rat and a hibernator the garden dormouse In Depressed Metabolism (Edited by Musacchi X J and J F Saunders) Elsevier 1969 119

ÅKESSON B J ELOVSON and G ARVIDSON Initial incorporation into a rat liver glycerolipids of intraportally injected (^{3}H) glycerol Biochem Biophys. Acta 1970 210 15

ALBRECHTSEN O K. Fibrinolyti activity i the organism. Acta physiol scand 1959 47 Suppl 165

ALEXANDER G.J and L M. KOPELOFF Fluctuations in serum holesterol levels i the monkey Amer J Med Sci 1969 257 24

ALM P L. CEGRELL B EHINGER and B FALCK, Remarkabl adrenergic nerves i the exocrine pancreas Z. Zellforsch 1967 83 178

ALOIA R C. and E T PENGELLEY Ultrastructure of the ventricular tissue of the hibernating ground squirrel Citellus lateralis in relation to the physiology of the hibernator's heart Comp. Biochem Physiol 1971 38 517

ANDJUS R.K. T CIRKOVIC N CUPERLOVIC J DAVIDOVIC V MAKOVIC USKOKOVIC and T VELIMIROVIC Brai metabolism and resistanc of hibernator (Citellus citellus) and the rat to different anoxi conditions including cardiac arrest i deep hypotherm Ann Acad Sci Fenn Ser A IV 1964 71 11

ANGELAKOS E.T K. FUXE and M.L. TORCHIANA Chemical and histochemical evaluation of the distribution of catecholamines i the rabbit and guinea-pig hearts. Acta physiol scand 1963 59 194

ANGELAKOS E T M.P KING J T MAHER and R BURLINGTON Adrenergl Innervation of the heart in hibernating and non-hibernating species Cryobiology 1971 8 307

ASDELL S A Patterns of Mammalian Reproduction Ithaca New York Comstock, 1946

ASTRA Kli lska laborationsmetoder Esselte Stockholm 1947

ASTRUP T Tissue activators of plasminogen Fed Proc 1966 25 42

ASTRUP T Blood coagulation and fibrinolysis in tissue ulture and tissue repair Biochem Pharmacol Suppl Pergamon London 1968 241

AXELROD J W ALBERS and C D CLEMENTE Distribution of catecholamine-O-methyltransferase in the nervous system and other tissues J Neurochem 1959 5 68

AXELROD J Purification and properties of phenylethanolamine-N-methyltransferase J Biol Chem 1962 237 1657

BALL E G Some energy relationships in adipose tissue Ann N Y Acad Sci 1965 131 225

BARAKAT M.Z. and R R. HASSANEIN Biochemical analysis and seasonal variation of ertai cow blood constituents. Zentralblatt Veterinaermed 1969 16 220

BARR L. M.M. DEWEY and W BERGER Propagation of action potentl ls and the structure of the exus l cardiac muscl J gen Physiol 1965 48 797

BARTELS H R SCHMELZLE and S ULRICH Comparative studies of th respiratory function of mammalian blood V Insectivora shrew mole and non-hibernating and hibernating hedgehogs. Resp Physiol 1969 7 278

BAUMBER J and A. DENYES Acetate-1-C^{14} metabolism of white fat from hamsters I cold exposur and hibernation Amer J Physiol 1963 205 905

BERTLER A A CARLSSON and E ROSENGREN A method for the fluorimetri determination of adrenaline and noradrenaline in tissues Acta physiol scand 1958 44 273

BIÖRCK, G and B W JOHANSSON Comparative studies on temperature effects upon the lectrocardiogram in some vertebrates Acta physiol scand 1955 34 257

BIÖRCK G B W JOHANSSON and H SCHMID Reactions of hedgehogs hibernating and non-hibernating to the inhalation of oxygen carbon dioxide and nitrogen. Acta physiol scand 1956a 37 71

BIÖRCK G B W JOHANSSON and S VEIGE Some laboratory data on hedgehogs hibernating and non-hibernating Acta physiol scand 1956b 37 281

BIÖRCK, G and B W JOHANSSON The influence of anoxia on the hypothermic electrocardiogram of hedgehog and man. Cardiologia 1957 30 344

BÖRCK, G B W JOHANSSON and I M, NILSSON Blood coagulation studies I hedgehogs in a hibernating and a non-hibernating stat   and in dogs hypothermic and normothermic Acta physiol scand 1962 56 334

BJÖRKLUND A. Monoamine-containing fibres in the pituitary neuro-intermediate lobe of the pig and rat Z. Zellforsch 1968 89 573

BJÖRKLUND A. and B. FALCK, Histochemical characteri ation of a tryptamine-like substance stored i cells of the mammalian adenohypophysis Acta physiol scand 1969a 77 475

BJÖRKLUND A   and B. FALCK, Pituitary monoamines of the cat with special reference to the presence of an unidentified monoamine-like substanc in the adenohypophysis. Z. Zellforsch 1969b 93 254

BJÖRKLUND A. B FALCK, F HROMEK Ch OWMAN and K.A. WEST Identification and terminal distribution of the tubero-hypophyseal monoamine fibr systems in the rat by means of stereotaxic and microspectrofluorimetric techniques Brain Res. 1970 17 1

BJÖRKLUND A. B FALCK and Ch. OWMAN Fluorescenc microscopic and microspectrofluorometric techniques for the cellular localization and haracterization of biogenic amines. In S A. Berson (ed) Methods of Investigative and Diagnostic Endocrinology vol 1 J E. Rall and I J Kopi (eds) The Thyroid and Catecholamines North Holland Publishing Company Amsterdam 1972

BLATT W.F   J WALKER and M, MAGER Tissue lactic dehydrogenase isozymes: Variation in rats during pr longed cold exposure Amer J Physiol 1965 209 785

BOERNER-PATZELT D   Das braune Fett der sogenannten Winterschlafdrüse des Igels Z. mikr -anat Forsch. 1958 63 5

BOY J   M. BONNAFE and J B   MAZET Détermination du cholestérol total par un   modification de la méthode de pearson son emploi par voi   manuell   et automatique Ann Biol clin 1960 18 669

BOYER S H   D C FAINER and E J WATSON-WILLIAMS Lactate dehydrogenase variant from human blood: Evidence for molecular subunits Science (N Y) 1963 141 642

BRAKMAN P   Fibrinolysis: A standardized fibri plate method and a fibrinolyti assay of plasminogen Scheltema and Holkema Amsterdam 1967

BURLINGTON R F   and J E WIEBERS Anaerobic glycolysi i   cardiac tissue from   hibernator and   non-hibernator as affected by temperature and hypoxia. Comp Biochem. Physi l 1966 17 183

BURLINGTON R.F   Gluconeogenesis i kidney cortex slices from cold-exposed rats and hamsters Comp. Biochem. Physiol 1966 17 1049

BURLINGTON R.F and G J KLAIN Effect of hypoxia on gluconeogenesis in the albino rat and thirteen-lined ground squirrel (Citellus tridecemlineatus). Comp Biochem. Physiol 1967 20 275

BURLINGTON R F and J H SAMPSON Distribution and activity of lactic dehydrogenase isozymes in tissues from a hibernator and non-hibernator Comp Biochem. Physiol 1968 25 185

BURLINGTON R.F J T MAHER and C M. SIDEL Effect of hypoxia on blood gases acid-base balance and in vitro myocardial function in a hibernator and non-hibernator Fed Proc 1969 28 1042

BURLINGTON R.F J A VOGEL T M. BURTON and I A SALKOVITZ Cardiac output and regional blood flow in hypoxic woodchucks Amer J Physiol 1971 220 1565

BUCHER T and M. KLINGENBERG Wege des Wasserstoff I der lebendigen Organization. Angew Chem. 1958 70 552

CAHN R.D N O KAPLAN L. LEVINE and E. ZWILLING Nature and development of lactic dehydrogenases. Science (Lond) 1962 136 962

CAHN R.D Cellular damage and the control of lactic dehydrogenase synthesis I cell cultures by oxidative metabolites. J Cell Biol 1963 19 (2) 12A.

CAHN R.D Developmental changes I embryonl enzyme patterns the effect of oxidative substrates on lactl dehydrogenase I beating hick embryonl heart cell cultures. Dev Biol 1964 9 327

CARLSSON L.A. and S LINDSTEDT The Stockholm study 1 The initial values for plasma lipids Acta med scand 1969 Suppl 493 1

CELLARIUS A Harmonia macrocosmica Amsterdam 1661

CHAFFEE R R J F L. HOCH and C P LYMAN Mitochondrial oxidative enzymes and phosphorylations I cold exposure and hibernation Amer J Physiol 1961 201 29

CHAFFEE R.R J J ALLEN Y CASSUTO and R SMITH Biochemistry of brown fat and liver in cold acclimatized hamsters. Amer J Physiol 1964 207 1211

CHAFFEE R.R.J E T PENGELLEY J R ALLEN and R.E. SMITH Biochemistry of brown fat and liver of hibernating golden-mantled ground squirrels (Citellus lateralis) Canad J Physiol Pharmacol 1966 44 217

CHALVARDJIAN A M. Fatty acids of brown and yellow fat I rats Biochem. J 1964 90 518

CHATFIELD P O A F BATTISTA C P LYMAN and J P GARCIA Effects of cooling on nerve conduction I a hibernator (golden hamster) and non-hibernator (albino rat) Amer J Physiol 1948 155 179

CHRISTIAN J.J Potassium deficiency in marmots during hibernation. Science 1961 134 390

CLARK, L.C. Jr Monitor and control of blood and tissue oxygen tensions Trans Amer Soc Artif Intern. Organs 1956 2 41

CLAUSEN G Acid base balance in the hedgehog Erinaceus europaeus L. during hibernation hypothermi cooling and rewarming Årb Univ Bergen, Mat Naturv Seri No 4 1966

CLAUSEN G and A ERSLAND The respiratory properties of the blood of the hibernating hedgehog Erinaceus europaeus L. Respir Physiol 1968 5 221

CORRODI H. and G JONSSON The formaldehyd fluorescence method for the histochemical demonstration of biogeni monoamines. J Histochem. Cytochem 1967 15 65

COVINO B G and J P HANNON Myocardial metaboli and electrical properties of rabbits and ground squirrels at low temperatures Amer J Physiol 1959 197 494

DAHLSTRÖM A K. FUXE M. MAYA-TU and B E.M. ZETTERSTRÖM Observations on the adrenergi innervation of the dog heart Amer J Physiol 1965 209 689

DAHLSTRÖM A. and K. FUXE Monoamines and the pituitary gland Acta endocr (Kbh.) 1966 51 301

DAVIDSON J K. M. ZIEGLER and R E HAIST Failure of guinea-pig antibody to beef insuli to neutralize coypu (nutria) insuli Diabetes 1968 17 8

DAVIDSON J K. M. ZIEGLER and R E. HAIST Failure of guinea-pig antibodies to beef insulin chicken insuli and od insuli to neutralize capybara insuli Diabetes 1969 18 212

DAVIES T W The monitoring of blood pressure during normal working day using an adaptation of an aneroid sphygmomanometer Clin Sci 1967 33 183

DAVIES W H Hibernation: Ecology and physiological ecology I Biology of bats (Edited by Wimsatt W.A) 1970 Vol. I Chap 8 265

DAWSON D M. T L GOODFRIEND and N O KAPLAN Lactic dehydrogenases functions of the two types rates of synthesi of the two major forms can be correlated with metabolic differentiation Science (N.Y) 1964 143 929

DENYES A. and J HASSETT A study of the metabolism of liver diaphragm and kidney I cold-exposed and hibernating hamsters. I Mammalian Hibernation (Edited by Lyman C P and A R Dawe) Bull Mus. Comp Zool (Harv) 1960 124 437

DENYES A. and J D CARTER Utilization of acetate-1-C^{14} by hepatic tissue from cold-exposed and hibernating hamsters. Amer J Physiol 1961 200 1043

DEWEY M.M. The structure and function of the intercalated disc in vertebrate cardiac muscle. I Comparative Physiology of the heart: Current trends. (Edited by McCann F V) Basel-Stuttgart 1969 Experientia Supplementum 15.

DIXIT P K. I P LOWE C B HEGGESTAD and A LAZAROW Insuli content of microdissected fetal islets obtained from diabeti and normal rats Diabetes 1964 13 71

DRASKOCZY P R and C P LYMAN Turnover of catecholamines during hibernation Pharmacologist 1965 7 167

DRASKOCZY P R and C P LYMAN Turnover of catecholamines in active hibernating ground squirrels. J Pharmacol exp Ther 1967 155 101

DRYER R.L and J R PAULSRYD Effect of arousal on ATP levels in bats. Fed Proc 1966 25 (4) 1293

DUBOIS R Autonarcose carbonicoacétonémique ou sommeil hibernal de la marmotte. C R. Soc Biol 1896 47 149

DWORKIN S and W H FINNEY Artifici l hibernation in the woodchuck (Arctomys monax) Amer J Physiol 1927 80 75

EDWARDS B A and K.A MUNDAY The function of brown fat i the hedgehog (Erinaceus europaeus) Comp Biochem Physiol 1969a 30 1029

EDWARDS B A. and K.A. MUNDAY Electrolyte metabolism in the hibernating hedgehog (Erinaceus europaeus) Comp Biochem. Physiol 1969b 31 329

EHINGER B B FALCK and B SPORRONG Adrenergi fibres to the heart and to peripheral vessels Bibl Anat 1967 8 35

EHINGER B B FALCK, H PERSSON and B SPORRONG Adrenergic and cholinesterase-containing neurons of the heart Histochemie 1968 16 197

ELIASSEN E The sodium and potassium contents of serum and urine in the hibernating hedgehog Årbok, Univ Bergen Mat Nat 1963 6

EPSTEIN C J and C B ANFINSEN The use of gel filtration i the isolation and purification of beef insuli Biochemistry 1963 2 461

ESTABROOK R.W and B SACKTOR α-Glycerophosphate oxidase of flight muscl mitochondria. J Biol Chem 1958 233 1014

FALCK B Observations on the possibilities of the cellular localization of monoamines by fluorescence method Acta physiol scand 1962 56 suppl 197 1

FALCK B N Å HILLARP G THIEME and A TORP Fluorescenc of catecholamines and related compounds condensed with formald hyd J Histochem. Cytochem. 1962. 10 348

FALCK B B LARSSON C MECKLENBURG E ROSENGREN and K. SVENAEUS On the presenc of second specific ell system i mammalian thyroid gland Acta physiol scand 1964 62 491

FALCK B and Ch OWMAN A detailed methodological description of the fluorescenc method for the cellular demonstration of biogeni monoamines Acta Univ Lund II 1965 7 1

FALCK, B Ch. OWMAN and N O SJÖSTRAND Peripherally located adrenergic neurons innervating the vas deferens and the seminal vesicle of the guinea-pig Experientia 1965 21 98

FALCK, B. and Ch. OWMAN 5-Hydroxytryptamine and related amines I endocrine cell systems Advanc Pharmacol 1968 6 211

FALCK, B Ch. OWMAN E ROSENGREN and N -O SJÖBERG Persisting high level of transmitter in uterine short adrenergic neurons following prolonged treatment with 17 β-oestradiol Acta endocr (Kbh) 1969 62 77

FALCK, B K.C. NIELSEN Ch. OWMAN H. PERSSON and B SPORRONG Adrenergic mechanisms in the development of hypothermic ventricular fibrillation in the isolated perfused heart of the cat Europ J Pharmacol 1972a 17 66

FALCK, B S. GÅRDMARK, G NYBELL Ch. OWMAN E ROSENGREN and N O SJÖBERG Ovarian influence on the content of norepinephrine transmitter in guinea-pig and rat uterus. Endocrinology 1972b In press

FALKMER S. and S. WILSON Comparative aspects of the immunology and biology of insulin Diabetologia 1967 3, 519

FALKMER S. Comparative endocrinology of the islet tissue In Diabetes (Edited by Östman J) Excerpta Medica Foundation Amsterdam 1969 55

FARNER D S Comparative physiology: photoperiodicity Ann Rev Physiol 1961 23 71

FAWCETT D W and C P LYMAN The effect of low environmental temperature on the composition of depot fat I relation to hibernation. J Physiol 1954 126 235

FEIST D D and W B QUAY Effects of ld acclimation and arousal from hibernation on brown fat lipid and protei I the golden hamster (Mesocricetus auratus) Comp Biochem Physiol 1969 31 111

FINE J H. N O KAPLAN and D KUFTINEC Developmental changes of mammalian lactic dehydrogenases. Biochemistry 1963 2 116

FISHER K.C and J F MANERY Water and lectrolyt metabolism I heterotherms In Mammalian Hibernation III (Edited by Fisher K.C et al) American Elsevier New York 1967 235

FLATT J P and E G BALL Studies on the metabolism of adipose tissue XV An evaluation of the major pathways of glucose catabolism as influenced by insulin and epinephrine J Biol Chem. 1964 239 675

FLEISCHMAN A I T HAYTON and M.L. BIERENBAUM Seasonal variations in serum ... in the young coronary male on controlled fat diets six-year cohort study Circulation 1967 36 Suppl II 11

FORSBERG A and H S S SARAJAS Studies on the metabolism of ^{14}C-labelled glucose I awake and hibernating hedgehogs Ann Acad Sci Fenn, Ser A, IV 1955 28 3

FREHN J L. Mitochondrial respiration in mammalian liver during cold exposure and hiber nation. Trans. Ill St Acad Sci 1966 59 342

FULLER R.W and J M. HUNT Inhibition of phenylethanolamine-N-methyltransferase by its product epinephrine Life Sci 1967 6 1107

GALSTER W.A and P MORRISON Seasonal changes I serum lipids and proteins in the 13-lined ground squirrel Comp Biochem Physiol 1966 18 489

GALSTER W A and P MORRISON Cyclic changes I carbohydrate concentrations during hibernation I the arctI ground squirrel Amer J Physiol 1970 218 1228

GERSHON M.D and E A. NUNEZ, Histochemical and radioautographic studies of serotonI and parafollicular cells I the thyroid gland of the prehibernating bat Endocrinology 1970 86 160

GOODFRIEND T L. and N O KAPLAN Induction of changes I the subunit composition of lactI dehydrogenase J Cell Biol 1963 19 (2.) 28A

GOODRICH C A. Acid-base balance I euthermI and hibernating marmots Amer J Physiol 1972 Submitted for publication.

GREEN D E α-Glycerophosphate dehydrogenase Biochem. J 1936 30 629

HAGGENDAL J An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline I plasma and tissues Acta physiol scand 1963 59 242

HÅKANSSON R L.-I LARSSON A NOBIN and F SUNDLER Tryptamine or tryptophylpeptides in endocrine cells of the mammalian adenohypophysI ? J Histochem Cytochem, 1972 I press

HANNON J P D A VAUGHAN and R HOCK Endogenous tissue respiration of the arctI ground squirrel as affected by hibernation and season J Cell Comp Physiol 1961 57 5

HANNON J P and D A VAUGHAN I itial stages of intermediary glucose catabolism in the hibernator and non-hibernator Amer J Physiol 1961 201 217

HANSON A and B W JOHANSSON Myocardial lactate concentration in guinea-pigs normothermI and hypothermI and hedgehogs I hibernating and nonhibernating stat Acta physiol scand 1961 53 137

HARDMAN M.J and D HULL Fat metabolism I brown adipose tissue I vivo J Physiol 1970 206 263

HARLAN W R and S.J WAKIL Synthesis of fatty acids I animal tissues I Incorporation of C^{14}-acetyl coenzyme A into variety of long chain fatty acids by subcellular particles J Biol Chem 1963 238 3216

HAWLEY P L. Energy sources in hibernator hearts pyridine nucleotide metabolism Proc Soc exp Biol (N Y) 1968 129 430

HAYWARD J S and C P LYMAN Nonshivering heat production during arousal from hibernation and evidence for the contribution of brown fat In Mammalian Hibernation III (Edited by Fisher K C et al) American Elsevier New York 1967 346.

HAYWARD J S The magnitude of noradrenaline induced thermogenesis in the bat (Myotis lucifugus) and its relation to arousal from hibernation Canad J Physi l Pharmacol 1968 46 713

HENDE van den C E MUYLLE and W OYAERT Enzyme activities of liver heart and skeletal muscles of the pig. Zbl Vet Med A 1968 15 135

HENRIQUES V and C HANSEN Vergleichend Untersuchung über die chemische Zusammensetzung des tierischen Fettes Skand Arch Physiol 1901 11 151

HEROUX O Climatic and temperature induced hanges in mammals Rev Canad Biol 1961 20 55

HIESTRAND W A W T ROCKHOLD F W STEMLER D E STULLKEN and J E WIEBERS The comparative hypoxic resistanc of hibernators and non-hibernators. Physiol Zool 1950 23 264

HOFFMAN R.A Terrestrial animals in cold: hibernators I Handbook of Physiology Sec 4 Adaptation to the Environment Amer Physiol Soc 1964 379

HOLLENBERG C H and A ANGEL R lation of fatty acid structure to lease and esterification of free fatty acids Amer J Physiol 1963 205 909

HONG S K. S H SONG P K. KIM and C S SUH Seasonal observations on the cardiac rhythm during diving I the Korean ama J appl Physiol 1967 23 18

HORWITZ, B A and L. NELSON Effect of temperature on mitochondri l respiration in hibernator (Myotis austroriparius) and non-hibernator (Rattus rattus) Comp Biochem. Physiol 1968 24 385

JACOBOWITZ D T COOPER and H B BARNER Histochemical and hemical studies of the localization of adrenergi and holinergi nerves i normal and denervated cat hearts. Circul Res 1967 20 289

JOEL C D The physiological rol of brown adipose tissue I Handbook of Physiology Sec 5 Adipose tissue (Edited by Renold A E and G F Cahill J) 1965 59

JOHANSEN K. Discussion following paper presented by Dr K. Fisher I Mammalian Hibernation II (Ed lainen P) Ann Acad. Sci Fenn Ser A, IV 1964 71

JOHANSSON B G Agarose gel electrophoresis Scand J lin Lab Invest 1972a Suppl 124 7

JOHANSSON B W Some biochemical and lectrocardiographical data on badgers. Acta Zool 1957 38 205

JOHANSSON B W Brown fat: A eview Metabolism 1959 8 221

JOHANSSON B W The effect of aconitine adrenalin and procaine and changes i the ionl concentration in the production of ventricular fibrillation i hibernator (hedgehog) nd non-hibernator (gui ea-pig) at different temperatures Cardiologi 1963 43 158

JOHANSSON B W Heart and irculation in hibernators In Mammalian Hibernation III (Edited by Fisher K.C et al) American Elsevier New York. 1967 200

JOHANSSON B W Electrocardiographi changes in depressed metabolism. I Depressed Metabolism (Edited by Musacchi X J and J F Saunders) Elsevier 1969a 313

JOHANSSON B W Temperature dependenc of cholinesterase and lactate dehydrogenase i the guinea-pig hedgehog and codfish. Acta physiol scand 1969b 77 1

JOHANSSON B W Effects of drugs on hibernation I The Pharmacology of Thermoregulation Symp San Francisco 1972b i press

JOHANSSON B W and T SVANBERG Unpublished observations

JØRGENSEN K. and P ASTRUP Standard bicarbonate its li i l significance and new method for its determination Scand J clin Lab Invest 1957 9 122

KARLSSON B W and E.J CARLSSON Levels of lactic and malic dehydrogenase isoenzymes i mammary gland milk and blood serum of the rat during pregnancy lactation and involution Comp Biochem Physiol 1968 25 949

KARLSSON B W and L.S PALMER Lacti dehydrogenase isozyme distribution i various tissue fractions of the developing mammalian liver Comp Biochem. Physiol 1971 38B 299

KAPLAN N Thermodynamics and mechanism of the phosphate bond I The Enzymes. New Yo 1951 II 55

KAPLAN N O and R D CAHN Lacti dehydrogenases and muscular dystrophy in the hicken. Proc nat Acad Sci (Wash) 1962 48 2123

KAPLAN N O Symposium on multiple forms of enzymes and control mechanisms. I Multipl forms of enzymes. Bact Rev 1963 27 155

KAPLAN N O Lactate dehydrogenase-structur and function Brookhaven Symp Biol 1964 17 131

KAYSER C Hibernation et hibernation artifici ll Rev Path. Gen Comp 1955 668 704

KAYSER C The Physiology of Natural Hibernation Pergamon Press Oxford 1961

KAYSER C and A MALAN Central nervous system in hibernation Experientia 1963 19 441

KAYSER C Stoffwechsel und Winterschlaf Helgoländer Wiss Meeresunters 1964 9 158

KAYSER C G VINCENDON R FRANK and A PORTE Some external (climatic) and internal (endocrine) factors in elation to production of hibernation Ann Acad Sci Fenn Ser A IV 1964 71 269

KITTO G B and A C WILSON Evolution of malate dehydrogenase in birds Science (N Y) 1966 153 1408

KODAMA A M. and N PACE Effect of environmental temperature on hamster body fat composition J appl Physiol 1964 19 863

KOJIMA T Studies on the variation of monoamine oxidase and acetylcholinesterase activities in the brain under some environmental conditions Jap. J Hyg 1966 20 351

KONTTINEN A M. RAJASALMI and H S S SARAJAS Fat metabolism of the hedgehog during the hibernating cycle Amer J Physiol 1964 207 845

KORNACKER M.S and E.G BALL Citrate cleavage in adipose tissue Proc. nat Acad. Sci (Wash.) 1965 54 899

KRAJL M.A A rapid microfluorometric determination of monoamine oxidase Biochem. Pharmacol 1965 14. 1683

KRISTOFFERSSON R Urea-levels in blood and tissues of hibernating and non-hibernating hedgehogs Nature (Lond) 1963 197 402

KRISTOFFERSSON R and A SOIVIO Hibernation in the hedgehog (Erinaceus europaeus L.) Ann Acad Sci Fenn Ser A IV 1964 82 3

KRISTOFFERSSON R A SOIVIO and P SUOMALAINEN Studies on the physiology of the hibernating hedgehog Ann Acad Sci Fenn Ser A IV 1965 92 3

LATNER A L. and A W SKILLEN Isoenzymes in biology and medicine Academic Press London 1968

LAUFBERGER V Versuche über di Insulinwirkung Z. ges exp Med 1924 42 570

LAURELL S A method for routine determination of plasma triglycerides. Scand J clin lab Invest 1966 18 668

LAURELL S and G TIBBLING Colorimetric micro-determination of free fatty acids i plasma. Cli Chim Acta 1967 16 57

LAYNE E 1 Methods in Enzymology (Edited by Colowick S P and N O Kaplan) Acad Press New York, 1957 Vol III, Section IV 451

LEVINS R Dormancy as an adaptive strategy Symp Soc exp Biol 1969 23 1

LIDICKER W Z, Jr and W H DAVIS Changes in spleni weight associated with hibernation in bats Proc Soc exp Biol (N Y) 1955 89 640

LINDBERG O ed Brown Adipose Tissue Elsevier 1970

LINDSAY D T Isozymic patterns and properties of lactate dehydrogenase from developing tissues f the hicken J exp Zool 1963 152 75

LINDY S and M. RAJASALMI Lactate dehydrogenase isozymes of chick embryo: response to variations of ambient oxygen tension Science (N Y) 1966 153 1401

LIU C -C J L. FREHN and A D LaPORTA Liver and brown fat mitochondrial response to cold in hibernators and non-hibernators J appl Physiol 1969 27 83

LOWENSTEIN J M The pathway of hydrogen in biosynthesis 1 Experiments with glucose-1 H^3 and lactate-2-H^3 J Biol Chem, 1961 236 1213

LOWRY V H N J ROSENBROUGH A L. FARR and R J RANDALL Protein measurement with Folin-phenol reagent J Biol Chem 1951 193, 265

LYMAN C P and A B HASTINGS Total CO_2, plasma pH and pCO_2 of hamsters and ground squirrels during hibernation Amer J Physl l 1951 167 633

LYMAN C P and D C BLINKS The effect of temperature on the isolated hearts of closely related hibernators and non-hibernators J cell comp Physiol 1959 54 53

LYMAN C P and R C OBRIEN Autonomic control of irculation during the hibernating cycl in ground squirrel J Physiol (Lond.) 1963 168 477

LYMAN C P Circulation in mammalian hibernation 1 Handbook of Physiology Sect 2 Circulation Vol III Amer Physl l Soc (Wash) 1965 1967

LYMAN C P Thermoregulation and metabolism in bats. I Biology of Bats (Edited by Wimsatt W A) 1970 Vol 1 Chap. 9 301

McDONOUGH J R and C G HAMES Influenc of rac sex and occupation on seasonal changes in serum cholesterol Amer J Epidemiol 1967 85 256

MAGER M. W F BLATT and R.W NEWMAN Loctic dehydrogenase isozymes: variations i the plasma of me exposed to cold J appl Physiol 1968 24 616

MARKERT C L. Lactat dehydrogenase isozymes: dissociation and recombination of subunits. Science (N Y) 1963 140 1329

MARSHALL J M. and J S WILLIS The effects of temperature on the membrane potentials i isolated atria of the ground squirrel Citellus tridecemlineatus J Physiol (Lond) 1962 164 64

MARSHALL, J M. Adrenergic innervation of the femal reproductive tract: Anatomy physiology and pharmacology Rev Physiol Biochem Exp Pharmacol 1970 62 5

MELANDER A E NILSSON and F SUNDLER Sympatheti activation of thyroid hormone secretion i mice Endocrinology 1972 1 press

MICHAEL C R and M. MENAKER The effect of temperature on the isolated heart of the bat Myotus lucifugus. J ll comp Physiol 1963 62 355

MILLONIG G Advantages of phosphate buffer for OsO_4 solutions I fixation J appl Physiol 1961 32 1637

MILSKY I.A R JINKS and G PERIUTTI The isolation and crystallization of human tissue J clin Invest 1963 42 1869

MOKRASCH L C H J GRADY and S GRISOLIA Thermogenic and adaptive me hanisms in hibernation and arousal from hibernation. Amer J Physiol 1960 199 945

MOLONEY P J and M. COVAL Antigenicity of insulin: Diabetes induced by specifi antibodies. Biochem J 1955 59 179

MORELAND J E Electronmicroscope studies of mitochondria I ardiac and kelet l muscl from hibernated ground squirrel Anat Rec 1962 142 155

MORIYA K. and S ITOH Regional and seasonal differences I the fatty acid omposition of human subcutaneous fat Int J Biometeor 1969 13 141

MUSACCHIA X J M. JELLINEK and T COOPER Effects of X irradiation during hibernation on tissue catecholamine contents Experientia 1963 19 418

MUSACCHIA X J and A V BRAIMATE Intestinal absorption I hamster and ground squirrel I ivo I Mammalian Hibernation III (Edited by Fisher K.C et al) American Elsevier New York. 1967 382

NELSON R.A H W WAHNER J D JONES and P E ZOLLMAN Urea and urine volume I bears The Physiologist 1971 14 201 (Abstr)

NIELSEN K.C and Ch OWMAN On the control of ventricular fibrillation during hypothermia. Commun Dept Anat Univ Lund 1965 6, 1

NIELSEN K.C and Ch OWMAN Control of ventricular fibrillation during induced hypothermia in cats after differential depletion of cardiac catecholamine stores with prenylamine (Segontin) Circulation Res 1967 21 45

NIELSEN K C Experiment l studies on the involvement of adrenergi mechanisms in the development of ventricular fibrillation during induced hypotherm Studentlitteratur Lund Sweden 1968 1

NIELSEN K C. and Ch OWMAN Differenc in cardiac adrenergi innervation between hibernators and non-hibernating mammal Act physiol scand 1968 Suppl 316 1

NILSSON I M. and M. PANDOLFI Fibrinolyti response of the vascular wall Thromb. Diathes haemorrh (Stuttg) 1970 Suppl 40 231

NOBLE R.P Electrophoretic separation of plasma lipoproteins I agarose gel J Lipid Res 1968 9 963

NUNEZ E A R P GOULD D W HAMILTON J S HAYWARD and S J HOLT Seasonal changes in the fi structure of the basal granular cells of the bat thyroid J Cell Sci 1967 2 401

OLSSON S -O R Unpublished results 1971

OWMAN Ch and N O SJÖSTRAND Short adrenergi neurons and catecholamine-contai-
ing cells I vas deferens and accessory male genital glands of different mammals
Z Zellforsch 1965 66 300

OWMAN Ch E. ROSENGREN and N -O SJÖBERG Origi of the adrenergi innervation
to the femal genital tract of the rabbit Life Sci 1966 5 1389

OWMAN Ch. and N -O SJÖBERG Adrenergic nerves in the femal genital tract of the
rabbit With remarks on holinesterase-contai ing structures Z. Zellforsch, 1966
74 182

PALEUS S and B W JOHANSSON Seasonal variations I cytochrome c content of hedgehog
brown adipose tissue Acta chem scand 1968 22 342

PALMER L.S and B W KARLSSON Lacti dehydrogenase isozyme distributions in various
ll types of the mammalian liver Canad J Biochem. 1969 47 1171

PANDE S V R PARVIN KHAN and T A VENKITASUBRAMANIAN Nicotinamide adenine
dinucleotid phosphate-specific dehydrogenases I relation t lipogenesis.
Biochim biophys Acta 1964 84 239

PANDOLFI M Localization of fibrinolytic activity I the developing rat eye Arch Ophthal
1967 78 512

PANDOLFI M. I M NILSSON B ROBERTSON and S ISACSSON Fibrinolyti activity
of human veins Lancet 1967 II 127

PAULSRUD J R and R L. DRYER Circum-annual changes I triglyceride fatty acids of bat brown
adipose tissue Lipids 1968 3 340

PAULSRUD J R K.G MANN and R L. DRYER A comparison of rat and bat malic dehydroge-
nase isoenzymes In Brown Adipose Tissue (Edited by Lindberg O) Elsevier New
York 1970 Chap 8 197

PENGELLEY E T The relation of external onditions to the onset and termination of hiberna-
tion. I Mammalian Hibernation III (Edited by Fisher K C et al) American
Elsevier New York. 1967 1

PENGELLEY E T and K.H KELLY Plasma potassium and sodium concentrations in active and
hibernating golden-mantled ground squirrels Citellus lateralis Comp Biochem.
Physiol 1967 20 299

PLAGEMANN P G W K.F GREGORY and F WROBLEWSKI Di lectrophoretisch trenn-
baren Lactat-dehydrogenasen des Säugetieres. III Einfluss der Temperatur auf die
Lactat-d hydrogenasen des Kaninchens Biochem. Z 1961 334 37

POCHE R Elektronenmikroskopische Untersuchungen zur Morphologie des Herzmuskels vom
Siebenschläfer während des aktiven und des lethargischen Zustandes Z. Zellforsch.
1959 50 332

POPOVIC V P Cardiac output i hibernating ground squirrels Amer J Physiol 1964 207 1345

PRECHT H J CHRISTOPHERSEN and H HENSEL, Temperatur und Leben, Springer-Verlag Berlin, 1955

PRUSINER S J R WILLIAMSON B CHANCE and B M. PADDLE Pyridine nucleotide changes during thermogenesi in brown fat tissue in vivo Arch Biochem 1968 123 368

PRUSINER, S B. CANNON and O LINDBERG Mechanisms controlling oxidative metabolism in brown adipose tissue I Brown Adipose Tissue (Ed. ed by Lindberg O) Elsevier New York. 1970 Chap 13 283

RASMUSSEN A T The so-called hibernating gland. J Morph 1923 38 147

RATHS P Ueber das Serum-Natrium-Kalium und Kalzium des winterschlafenden und hypothermischen Hamsters Z Biol 1962 113 173

REBEL, G J D WEILL P MANDEL and C KAYSER Y a-t-il formation de glycogène à partir des acides gras hez les hibernants en sommeil hivernal? C R [illegible] Biol (Paris) 1960 154 2118

REBUP C and I LUNDQUIST Non-specific eaction of urrent glucose oxidase preparations with glycogen and its application for glycogen determinations in tissue Acta pharmacol (Kbh.) 1967 25 41

ROGNSTAD R. and J KATZ Gluconeogenesis in the kidney ortex Effects of D-malate and amino-oxyacetate Biochem. J 1970 116 483

ROMANS R.G The preparation and chemistry of crystalline insulin Recent Progr Hormone Res. 1954 10 241

RONDININI R G GANDOLFI and E. TURCHETTO Variations in the fatty acids of the lipids present in the subcutaneous tissue of the "Rhinolophus Ferrum Equinum" during the hibernation. Riv Biol 1962 55. 215

ROSENGREN E. and N O SJÖBERG The adrenergic nerve supply to the femal reproductive tracts of the cat Amer J Anat 1967 121 271

ROSENQVIST T H and M L. ZIMNY Modification of the cell surface and the muscular triad i the heart of hibernator The Physiologist 1969 12 343

ROSENQVIST T H Ultrastructural changes i the plasma membrane and sarcoplasmic reticulum of myocardial cell during hibernation. Cryobiology 1970 7 14

SACKTOR B A biochemical basi of flight muscle activity Proc. Ciba Fdn Coll Ageing 1959 12 138

SALTHE S.N Comparative catalyti studies of lactic dehydrogenases in the amph ronmental and physiological correlations. Comp Biochem Physiol 393

SAMUEL P S LIEBERMAN F S SHMASE E MEILMAN and G TOUFEXIS Variation of total serum cholesterol concentration I patients with atherosclerosi Amer J lin Nutr 1970 23 178

SCHALES O and S S SCHALES Simple and accurate method for determination of chloride I biological fluids J Biol Chem 1941 140. 879

SENTURIA J B S STEWART and M. MENAKER Rate-temperatur relationships in the isolated hearts of ground squirrels. Comp Biochem Physiol 1970 33 43

SEVERINGHAUS J W and A F BRADLEY Electrodes for blood pO_2 and pCO_2 determination J appl Physiol 1958 13 515

SIGGAARD ANDERSEN O S and W EGSBAEK Hypotermiens indvi kning på syrebas-status has hibernerend flagermus Ugeskr Laeg 1962 124, 929

SIMMONDS R C E C LARKIN and F ULVEDAL Lactic dehydrogenase activity in hibernating and active mexican ground squirrels (Spermophilus mexicanus) exposed to hypertoxic and normoxi tmosphere Hibernation-Hypothermia IV Symposium 1971 4 (Abstr)

SJÖBERG N O Th adrenergic transmitter of th female reproductive tract: Distribution and functional changes Acta physiol scand 1967 Suppl 305 1

SJÖBERG N -O Considerations on the cause of disappearance of the adrenergic transmitter I uterine nerves during pregnancy Acta physiol scand 1968a 72 510

SJÖBERG N O Increase I transmitter content of adrenergi nerves in the reproductive tract of female rabbits after oestrogen treatment Acta endocr (Kbh) 1968b 57 405

SJÖSTRAND N O The adrenergic innervation of the vas deferens and the accessory mal genital glands Acta physiol scand 1965 65 Suppl 257 1

SKEGGS L.T J An automatic method for colorimetric analysis Amer J clin Path. 1957 28 311

SMALLEY R C and R L DRYER Brown fat i hibernation I Mammalian Hibernation III. (Edited by Fisher K.C et al) American Elsevier New York. 1967 325

SMALLEY R L J J MINOR and L. ROWE The composition and metabolism of developing brown and whit fats Lipids 1970 5 231

SMITH L F Species variation i the amino acid sequence of insuli Amer J Med 1966 40 662

SMITH R E and R J HOCK, Brown fat: thermogeni effector of arousal i hibernators Science 1963 140 199

SMITH R E Thermogenesi and thyroid action Nature 1964 204 1311

SMITH R E and B A HORWITZ Brown fat and thermogenesis. Physiol Rev 1969 49 330

SOIVIO A. Hibernation in the hedgehog (E inaceus europaeus L.) the distribution of blood the size of the spleen and the hematocrit and hemoglobin values during the annual and hibernating cycles Ann Acad Sci Fenn A IV 1967 110 1

SOUTH F E Rates of oxygen consumption and glycolysis of ventricl and brai slices obtained from hibernating and non-hibernating mammals as function of temperature Physiol Zool 1958 31 6

SOUTH F E, and H JEFFAY Alterations in serum protein of hibernati g hamster Proc Soc exp Biol (N Y) 1958 98 885

SOUTH F E Hibernation temperature and rates of oxidative phosphorylation by heart mitochondria Amer J Physiol 1960a 198 463

SOUTH F E Some metabolic specializations i tissues of hibernating mammals I Mammalian Hibernation (Edited by Lyman C P and A R Dawe) Bull Mus Comp Zool (Harv) 1960b 124 475

SOUTH F E and W A HOUSE Energy metabolism i hibernation In Mammalian Hibernation III (Edited by Fisher K.C et al) American Elsevier New York, 1967 305

SPENCER W.A and G DEMPSTER The lipids of mouse brown fat Canad J Biochem 1962 40 1705

SPENCER W.A E I GROODUMS and G DEMPSTER The glyceride fatty acid composition and lipid content of brown and whit adipose tissue of the hibernator Citellus lateralis. J Cell Physiol 1966 67 431

SUOMALAINEN P Production of artificial hibernation. Nature 1938 142. 1157

SUOMALAINEN P and A.-M. HERLEVI The hibernating gland and the alarm reaction Arch. Soc. Zool Botan Fenn Vanamo 1950 5:2. 72

SUOMALAINEN P Proc Finn Acad Science and Letters, Helsinki 1954

SUOMALAINEN P and V J UUSPÄÄ Adrenaline/noradrenaline ratio in the adrenal glands of the hedgehog during summer activity and hibernation. Nature 1958 182. 1500

SUOMALAINEN P and P L. SAARIKOSKI The content of non-esterified fatty acids and glycerol i the blood of the hedgehog during the hibernation period Experientia 1967 23 457

TAKI T and T TAKETOMI Lipid and fatty acid composition of brown adipose tissue i rabbit Jap J exp Med 1969 39 471

TASHIMA L S S J ADELSTEIN and C P LYMAN Radioglucose utilization by active hibernating and arousing ground squirrels Amer J Physiol 1970 218 303

TEPPERMANN H M, and J TEPPERMANN H[illegible] shunt and adaptive lipogenesis Diabetes 1958 7 478

TEPPERMANN J and H M. TEPPERMANN Metabolism of glucose-1-C^{14} and glucose-6-C^{14} by liver slices of refed rats. Amer J Physiol 1961 200 1069

THOMAS C B H W D HOLLJES and F G EISENBERG Observations on seasonal variations in total serum cholesterol level among healthy young prisoners Ann Intern Med 1961 54 413

THORELL J I Use of immunosorbent in the preparation of an "antigenfree plasma An adequate diluent for standards i adioimmunoassay Clin chim Acta 1968 22 579

THORP J M. Effects of seasonal variation on lipid metabolism in animal and man Biochem Soc Symp 1963 24 163

TODD A S The histological localisation of fibrinolysi activator J Path Bact 1959 78 281

TWENTE J A and J W TWENTE pH and pCO_2 of blood of ground squirrels after known periods in hibernation Amer Zool 1964 4 296

TWENTE J A and J W TWENTE Concentrations of *l* lactate in the tissues of Citellus lateralis after known intervals of hibernating periods J Mammal 1968 49 341

UMAHARA Y Light and electron microscopic studies on the brown adipose tissue in the bat Arch Histol Jap 1968 29 459

UTTER M.F and K. KURAHASHI Mechanism of action of oxalocetate carboxylase J biol Chem 1954 207 821

UUSPAÄ, V J The catecholamine ontent of the brain and heart of the hedgehog (Erinaceus europaeus) during hibernation and in an active state Ann Med exp Fenn 1963a 41 340

UUSPAA, V J Effects of hibernation on the noradrenaline and adrenaline contents of the adrenal glands i the hedgehog Ann Med exp Fenn 1963b 41 349

WEINER J D and E P STEYN-PARVE The metabolism of glucose i normal and thiamine-deficient pigeons I The conversion of glucose into glycogen fatty acids and respiratory arbon dioxide Biochim biophys Acta 1959 35 473

WELLS H J M. MAKITA W W WELLS and P H KRUTZSCH A comparison of the lipid composition of brown adipose tissue from mal and femal bats (Myotis lucifugus) during hibernating and non-hibernating seasons. Biochem biophys. Acta 1965 98 269

WHITTEN B K. and G J KLAIN Prot i metabolism in hepati tissue of hibernating and arousing ground squirrel Amer J Physiol 1968a 214 1360

WHITTEN B.K. and G.J. KLAIN NADP specific dehydrogenases and [illegible]

[illegible] the hibernator. Comp. Biochem. Physiol. 1968, [illegible]

WHITTEN B.K. Regulation of heart [illegible] in the [illegible]

[illegible] IV Symposium Snowmass Aspen Colorado. [illegible]

WILKINSON J.H. Isoenzymes. E. and F.N. Spon Ltd. London [illegible]

WILLIS J.S. Cold adaptation of [illegible] of tissues of hibernating mammals. In Mammalian Hibernation III (Edited by Fisher K.C. [illegible] American Elsevier [illegible] 1967 356.

WILSON A.C. R.D. CAHN and N.O. KAPLAN Functions of the two forms of [illegible] dehydrogenase in the breast muscle of birds. [illegible] 19[illegible]

VINCENT DE BEAUVAIS Speculum Ma[illegible] [illegible] [illegible] 1470 [illegible] [illegible]

WIRSEN C. Adrenergic innervation of adipose tissue examined by fluorescence microscopy Nature (Lond.) 1964 [illegible]

WIRSEN C. and B. HAMBERGER Catecholamines in brown fat [illegible] 625

YALOW R.S. and S.A. BERSON Immunoassay of endogenous plasma insulin [illegible] Invest. 1960. 39 1157

ZAJUSZ, K. Erinaceus europaeus in a hedgehog and its changes [illegible] Endok. pol. 1957 8 11.

ZIMNY M.L. Metabolism of some carbohydrate and phosphorus compounds during hibernation in the ground squirrel. J. cell. comp. Physiol. [illegible] 271

ZIMNY M.L. and J.E. MORELLA [illegible] Mitochondrial granules and succinic dehydrogenase in the heart of a hibernator. Canad. J. Physiol. Pharmacol. 1968, 46 [illegible]

ZIMNY M.L. M. SHERMAN and C. ROMANO [illegible] of the intercalated disc during hypothermia in the rat and the ground squirrel Cryobiology 1968 4 317

ZIRM, K.L. Ein Beitrag zur Kenntnis des natürlichen Winterschlafes und seiner regulierenden Wirkstoffe I. Z. Naturforsch. 1956 11b [illegible]

ZÖLLNER N. and K. KIRSCH Über die quantitative Bestimmung von Lipoiden (Mikromethode) mittels der vielen natürlichen Lipoiden (allen bekannten Plasmalipoiden) gemeinsamen Sulfophosphovanillin Reaktion Z. ges. exp. Med. 1962 135 545.

Sonetto Dimostrativo
Sopra il Concerto Intitolato La
PRIMAVERA
DEL SIG.re D ANTONIO VIVALDI

A [illegible]
B [illegible]
C [illegible]
[illegible]

D [illegible]
[illegible]
E [illegible]
[illegible]

F [illegible]
[illegible]
[illegible]

G [illegible]
[illegible]
[illegible]

Concerto

LA PRIMAVERA

I

Allegro A

Sonetto Dimostrativo
Sopra il Concerto Intitolato
L'ESTADE
DEL SIG.re D ANTONIO VIVALDI

A [illegible]
[illegible]
B [illegible]
C [illegible]

D [illegible]
[illegible]
E [illegible]
[illegible]

F [illegible]
[illegible]
[illegible]

G [illegible]
[illegible]
[illegible]

Concerto No. 2

L'ESTATE

A

Sonetto Dimostrativo
Sopra il Concerto Intitolato
L'AUTUNNO
DEL SIG.re D. ANTONIO VIVALDI

A.
B.
C.

D.

E.
F.

G.
H.

Concerto
L'AUTUNNO

Sonetto Dimostrativo
Sopra il Concerto Intitolato
L'INVERNO
DEL SIG.re D ANTONIO VIVALDI

A
B
C
D

E
F
G

H
I
L

M
N

Concerto 4
L'INVERNO

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 381

From the Institute of Medical Physiology C
University of Copenhagen Denmark

Acetylcholine-induced Ion Transports Involved in the Formation of Saliva

by

OLE HOLGER PETERSEN



Copenhagen 19

Forsvaret finder sted
torsdag den 9. november 1972 kl. 14.00 præcis i
Universitetets annex-auditorium A, Studiestræde 6 (over gården)

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 381

From the Institute of Medical Physiology C
University of Copenhagen Denmark

Acetylcholine-Induced Ion Transports Involved in the Formation of Saliva

by

OLE HOLGER PETERSEN



Copenhagen 1972

Denne afhandling er i forbindelse med omstående tidligere publicerede afhandlinger af det lægevidenskabelige fakultet ved Københavns Universitet antaget til offentlig at forsvares for den medicinske doktorgrad

Kobenhavn, den 23 maj 1972
Poul Kruhoffer
dekan

The manuscript of the present monograph was submitted to the Faculty of Medicine of the *University of Copenhagen May* 1971 and was accepted as a doctorate thesis on the 23rd of May 1972.

Previously Published Papers

A) O H Petersen J H Poulsen and N.A. Thorn SECRETORY POTEN TIALS SECRETORY RATE AND WATER PERMEABILITY OF THE DUCT SYSTEM IN THE CAT SUBMANDIBULAR GLAND DURING PERFUSION WITH CALCIUM-FREE LOCKE s SOLUTION Acta physiol scand. 1967 *71* 203-210.

B) O H. Petersen, SOME FACTORS INFLUENCING STIMULATION INDUCED RELEASE OF POTASSIUM FROM THE CAT SUBMAN DIBULAR GLAND TO FLUID PERFUSED THROUGH THE GLAND J Physiol (Lond.) 1970 *208* 431-447

C) O.H. Petersen, THE EFFECT OF DINITROPHENOL ON SECRE TORY POTENTIALS SECRETION AND POTASSIUM ACCUMULA TION IN THE PERFUSED CAT SUBMANDIBULAR GLAND Acta physiol. scand 1970 *80* 117 121

D) O H Petersen THE DEPENDENCE OF THE TRANSMEMBRANE SALIVARY SECRETORY POTENTIAL ON THE EXTERNAL POTASSIUM AND SODIUM CONCENTRATION J Physiol (Lond) 1970 *210*- 205-215

E) O.H Petersen THE IMPORTANCE OF EXTRACELLULAR SODIUM AND POTASSIUM FOR ACETYLCHOLINE EVOKED SALIVARY SECRETION Experientia 1970 *26* 1103-1104

F) O H. Petersen, SECRETORY TRANSMEMBRANE POTENTIALS IN ACINAR CELLS FROM THE CAT SUBMANDIBULAR GLAND DURING PERFUSION WITH A CHLORIDE FREE SUCROSE SOLUTION Pflügers Arch 1971 *323* 91 95

G) O H. Petersen, FORMATION OF SALIVA AND POTASSIUM TRANSPORT IN THE PERFUSED CAT SUBMANDIBULAR GLAND J Physiol (Lond.) 1971 *216* 129 142.

Reference to these papers in the text is made by means of the let ters A-G.

Contents

Introduction

During resting conditions most salivary glands only secrete little or do not secrete at all. Electrical activity in the parasympathetic nerves supplying the glands results in copious secretion of saliva. The transmitter being released from the post-synaptic nerve endings is acetylcholine (ACh) (Emmelin 1967)

The most important part of the salivary gland with respect to fluid transfer from blood to lumen is the acinus (Burgen 1967) The role of the duct system in the glandular salt and water transport is mainly to reabsorb NaCl in excess of water and to secrete K^+ and HCO_3^- (Thaysen Thorn & Schwartz 1954 Thaysen 1960, Young & Schögel 1966 Petersen & Poulsen 1967a, Knauf & Frömter 1970a, Martin & Young 1971 and Young & Martin 1971) It is furthermore now known that the acinar primary secretion as predicted by Thaysen et al (1954) has the same tonicity as plasma and that it has a plasma-like Na^+ K^+ and Cl^- concentration (see p 28 for some exceptions to this rule) (Young & Schögel 1966 Martinez, Holzgreve & Frick 1966 and Mangos, Braun & Hamann 1966) It is probable that the concentrations of Mg^{2+} Ca^{2+} and inorganic phosphorus are considerably lower in the acinar secretion than in plasma (Nielsen & Petersen 1970)

The mechanisms responsible for the formation of the primary secretion are largely unknown. Two significant discoveries may however help us to understand how the secretory machinery is started by the action of ACh on the contraluminal acinar cell membrane. Lundberg (1955) discovered the transmembrane secretory potential in acinar cells from the salivary glands and Burgen (1956) discovered that salivary gland cells lose potassium to the blood and the saliva, when they are stimulated to secrete.

The aim of the experimental work to be discussed in this thesis was to find the mechanism underlying these two fundamental phenomena and to attempt a description of the individual steps leading to the formation of the acinar secretion.

Two shorter reviews of the same experimental work have already appeared (Petersen 1971 c,d)

I

The secretory potential difference across the contraluminal acinar cell membrane.

How does ACh activate the acinus by acting on the contraluminal cell membrane? The most thoroughly investigated synapse with ACh as transmitter is probably the neuro-muscular junction in striated muscle. ACh acts on the striated muscle end-plate by increasing the permeability to Na and K^+ and therefore depolarizes the cell membrane. This end plate potential is a graded response which above a certain threshold will evoke an ,all or none propagating action potential. There is little doubt that the striated muscle action potential is a requisite for muscular contraction under normal physiological conditions (Sten-Knudsen 1960) The results of the investigations into the mechanism of action of ACh on the end-plate have been essential for our understanding of how ACh initiates muscular contraction. It is therefore understandable that it was expected that studies of the mechanism of action of ACh on the salivary gland cell membranes could give important information pertinent to the problem of how salivary secretion is evoked.

Electrical potentials in salivary glands.

Bayliss and Bradford (1886) showed that salivary glands when stimulated to secrete produced electrical currents. Before the introduction of the glass micro-electrode to electrophysiology it was impossible however to explain the origin of these currents.

The first results of measurements of membrane potentials in salivary glands were published by Anders Lundberg (1954 1955) The acinar resting membrane potential in the cat submandibular gland was low compared with resting membrane potentials in nerve and muscle cells. Stimulation of the glands to secrete by electrical stimulation of the parasympathetic nerves to the gland or by intra-arterial injections of ACh adrenaline or pilocarpine increased markedly the intracellular negativity This stimulation-induced increase in membrane potential was named the secretory potential

In the cat submandibular gland other types of secretory potentials were also described, probably originating from other cell types. These other secretory potentials (type II and III) will not be considered here The type I secretory potential originating in the acinar cells was also described in the cat sublingual gland (Lundberg 1957a) in the dog submandibular gland (Imai 1965a) and in the cat parotid gland (Fritz & Bothelho 1969b) In the two latter cases the amplitude of the secretory potential was somewhat smaller than in the cat submandibular and sublingual glands. Also in the rat submandibular gland secretory potentials have been demonstrated (Schneyer and Yoshida 1969) Here the secretory potential may consist of a small depolarization, a small depolarization followed by a small hyperpolarization or a small hyperpolarization. Other exocrine glands have also been investigated. Hisado and Botelho (1968) demonstrated secretory potentials in the cat lacrimal gland and in the mouse exocrine pancreas Dean and Matthews (1968) found depolarizing secretory potentials.

Secretory potentials in exocrine glands may be characterized in the following way

1) There is a relatively long latency (about 300 msec) This has been discussed in detail by Creed & Wilson (1969)
2) The rate of polarization is relatively low (100-200 mV/sec.)
3) The amplitude of the secretory potential depends on the voltage of the stimulus (number of activated nerve fibers) and on the frequency of stimulation.

Table I

I tracellular concentrations of K^+ Na and Cl (meq/kg cell water) and their equil brium potentials (mV) across the contraluminal cell membranes during resting cond tions.

		$[K]_i$	E_K	$[Na]_i$	E_{Na}	$[Cl]_i$	E_{Cl}
Rat	submandibular gland*	194	+91	15	+52	34	+41
Cat	submand bular gland£	115	+90	47	+32	72	+15
	subl ngual gland	147	+97	53	+29	80	+12
Dog	submandibular gland	117	85	3	+95	62	+23

The intracellular concentrations have been calculated from Schneyer & Schneyer's (1965a) figures for electrolyt content, water content, and inul n space tissue slices. The equ librium potential have been calculated on the basis of these figures and the e tracellular oncentr tions which wer (meq/l) K^+ 6.5 Na 149 and Cl 158

£ All figures are from Burgen & Emmel n (1961) p 205

All figures are from Lundberg (1958)

The intracellular concentrations have been calculated by Imai (1965b) The equilibrium potentials wer calculated on the basis of these figures and the figures for the extracellular concentrations, which were (meql) K^+ 4.0, Na 150 and Cl^- 148

4) The membrane potential does not return to the resting level until cessation of the stimulation.

Table II

The magnitude of the resting membrane potentials in acinar cells f om d fferent salivary glands

Rat	submandibular gland (in vivo)*	+23 mV ± 1.0(S.E.) (n=43)
	parotid gland (in vivo)*	+20 mV ± 2.4(S E.) (n 10)
Cat	submandibular gland (in vivo)[£]	+22 mV (n 100)
	submand bular gland (perfused with Locke s solution)	+20.7 mV ± 0.5 (S.E.) (n=123)
	sublingual gland (in vivo)[α]	+33 mV (n 100)
	parotid gland (n vivo)[β]	+20 8 mV ± 7 1 (S.D) (n=234)
Dog	submandibular gland (in vivo)[μ]	+42 mV ± 7 (n=150)
	submandibular gland (perfused with Ringer's solution)*	+40 mV ± 8 (n=200)
Syrian hamster	submandibular gland (in vivo)[x]	+22 mV ± 2

	Schneyer & Schneyer (1965b)	β	Fritz & Botelho (1969a)
£	Lundberg (1955)	μ	Imai (1965a)
	Petersen (1970c)	**	Yosh mur & Imai (1967)
α	Lundberg (1957a)		Henriques & Sperling (1966)

The resting membrane potential.

Table I shows figures for the intracellular concentrations of some important ions during resting conditions. The calculated diffusion potentials for these ions are also included. The figures in table I represent results of experiments carried out on homogenates of salivary glands in which there are many cell types. Although the acinar cells dominate the salivary glands (B G) the figures must be regarded with some scepsis. This is true in particular for the figures for the intracellular Na concentration. Even slight variations in the glandular inulin space would greatly influence the calculated intracellular Na concentration This is, of course a general problem. In the salivary glands there is the additional problem that it is unknown to which extent inulin penetrates into the transcellular water compartment (the duct system) (Schneyer & Schneyer 1962) Table II shows figures for the magni tude of the resting membrane potential in acinar cells from different salivary glands.

The resting membrane potential is close to the Cl equilibrium potential This is also so in the human red cell (Lassen & Sten-Knudsen 1968) In the case of the red cell it is the distribution of Cl that determines the membrane potential This is not so certain for the acinar cells. Even dramatic

alterations in the extracellular Cl concentration have failed to influence the membrane potential significantly (Lundberg 1957c, Petersen & Poulsen 1969 and F) In the experiments reported in these papers, which were not concerned with the mechanism underlying the resting membrane potential membrane potentials were never measured until 5-10 min had elapsed after changing the composition of the perfusion fluid It would be of great interest to study the membrane potential in the period immediately following the alterations of the ionic composition of the perfusion fluid The dependence of the resting membrane potential on the extracellular K^+ concentration is not very marked (D) and replacement of extracellular Na by tetraethylammonium does not alter the resting potential markedly It is at present obscure which parameters determine the resting acinar membrane potential

From the figures listed in tables I and II it is obvious that large electrochemical gradients for K^+-efflux and Na-influx exist. The distribution of Na and K^+ across the contraluminal cell membrane must therefore be upheld by an energy-requiring mechanism extruding Na and accumulating K^+ (see discussion in B)

The studies of Lundberg into the mechanism underlying the secretory potential in the cat sublingual gland.

The resting membrane potential in the tubular cells (corresponding to the acinar cells in the submandibular gland) was about 30 mV (the cell inner being negative with respect to the outside) Maximal stimulation changed the membrane potential to about -60 mV The potential difference between the intracellularly located tip of a microelectrode and a reference electrode placed on the surface of the gland represented the membrane potential If the tip of the microelectrode was placed in the tubular lumen the secretory potential seen after stimulation did not differ much from that recorded with the microelectrode in the intracellular position (see also p 29) Lundberg therefore concluded that the increased intracellular negativity seen after stimulation could be explained by a hyperpolarization of the contraluminal membrane and that the potential difference across the luminal membrane did not change markedly (Lundberg 1957a)

The important question was now whether the hyperpolarization of the contraluminal cell membrane could be explained by the existence of ion movements along electrochemical gradients (passive transport) or by assuming the existence of energy requiring ion transport processes (active transport) From the figures given in tables I and II it is obvious that the

only downhill ion movement which could result in hyperpolarization is an efflux of K^+ If one assumes that ACh through its interaction with the cell membrane evokes an increased permeability to K^+ the result would be K^+ efflux and a tendency for the membrane potential to approach the K^+ equilibrium potential (E_K) Since the membrane potential is much lower than E_K there would be a hyperpolarization. If however we were able to increase the membrane potential to a level above 100 mV there would be a tendency for K^+ to move into the cells and the result of an increased K^+ permeability would be a depolarization.

Lundberg (1957b) tried to do this experiment by employing the double-barrelled micro-electrode, that had been introduced in electrophysiology by Coombs, Eccles & Fatt (1955) Through one barrel hyper or depolarizing current could be applied whereas the membrane potential was recorded through the other barrel. Lundberg concluded from the results of this type of experiments that the magnitude and polarity of the secretory potential was independent of the level of the resting membrane potential when this ranged between 0 and 100 mV Unfortunately there is no quantitative treatment of the results, only one typical experiment is shown. The result from this typical experiment (Lundberg 1957b Fig. 1) is that the secretory potential evoked when the resting potential (RP) was 100 mV is of a somewhat smaller amplitude than that evoked when the RP was 30 mV The secretory potential evoked at a RP of 10 mV is somewhat larger than the normal one. Unfortunately one cannot be certain that the changes in membrane potential produced by the application of current through the one barrel of the micro-electrode really were as large as measured in these experiments. The tubular cells of the sublingual gland as well as the acinar cells of the submandibular gland are densely packed with organelles (Leeson 1967) Lundberg (1957b) indeed admitted that it was possible that part of the applied current passed through intracellular membranes so that part of the recorded voltage drop might have taken place within the cell rather than over the cell membrane proper If this were true the interpretation of the results of these crucial experiments would change significantly

It may be relevant to mention here that similar experiments carried out on the abdominal ganglion of *Aplysia* led to the assumption of the existence of an active ion transport being responsible for a hyperpolarization. Recently this concept has been challenged (Kehoe & Ascher 1970) (see also p. 22)

Lundberg concluded that the secretory potential could be explained by the existence of an active electrogenic pump mechanism Two possibilities existed An active cation extrusion or an active anion uptake. Lundberg

(1957b) was in favour of the latter possibility since in that case the ion transport would be in the same direction as the secretory process itself. Lundberg (1957c) consequently carried out experiments in the perfused sublingual gland in which the effect of replacement of Cl by NO_3 was looked at. The amplitude of the secretory potentials was shown to be definitely reduced after replacement of Cl by NO_3. The secretory rate was also severely reduced. Lundberg (1957c) therefore concluded that an active electrogenic Cl uptake was responsible for the existence of the secretory potential. It was felt likely that this Cl transport might be important in the formation of the saliva. A comparison between the rate of Cl transport carried out by this alleged pump and the rate of Cl excretion in the saliva was therefore undertaken. In order to determine the strength of the Cl current it was necessary to know the membrane conductance during stimulation. The calculation of the membrane resistance in the tubular cells of *the sublingual gland was, among other things, based on a core-conductor* model of the sublingual tubule. The luminal compartment (saliva) was regarded as the low resistance core while the tubular cells were regarded as the high resistance wall. Lundberg assumed that when current was sent between a microelectrode placed in the luminal compartment and an external electrode there would be no current flow through adjacent junctional cell membranes in the sublingual gland tubule, that is each cell would be an electrically insulated unit as for example is known from nerve cells. This assumption is probably incorrect. Loewenstein (1966) has shown that generally epithelial cells have very permeable junctional membranes with much lower specific resistance than that of the surface cell membranes. Lundberg's calculation gave as a result that the specific resistance of the contraluminal cell membrane was 18 Ωcm^2 during resting conditions. During stimulation the value was only 9 Ωcm^2. Having finally calculated the total area of contraluminal cell membrane it was possible to conclude that the amount of current needed from an external battery to equal the secretory potential could easily explain the rate of Cl excretion into the saliva. Lundberg therefore suggested that the active Cl transport was the essential (perhaps the one) active process in the formation of the saliva. For electro-static reasons Na would follow Cl and to maintain isosmolarity water would come along.

Lundberg interpreted the secretory potential as an essential phenomenon in the secretory process, namely as the electrical sign of the active chloride transport responsible for the formation of the saliva.

The studies of Imai on the dog submandibular gland

The type I secretory potentials first described by Lundberg are derived from the acinar cells. When looking through a dissecting microscope at high magnification Lundberg was able to see the micro-electrode entering an acinus. In those cases stimulation always evoked type I responses. Imai (1965a) confirmed this. After having obtained a type I response the punctured cell was marked by electrophoretic application of dye The subsequent histologic analysis identified the site of the tip of the microelectrode as an acinar cell This has been successfully repeated by Henriques & Sperling (1966) and Fritz & Botelho (1969a) Imai (1965a) measured the resistance between the cell interior and the external medium and found it to be about 18 MΩ during resting conditions and about 14 MΩ during stimulation. The comparable values of Lundberg were 2.0 MΩ and 1 8 MΩ.

In order to characterize the mechanism underlying the secretory potential Imai undertook a number of experiments where the effect of various ionic replacements on the magnitude of the secretory potentials was tested. It was important that Imai was able to show that the amplitude of the secretory potential was reduced after augmentation of the extracellular K^+ concentration (Imai 1965b Yoshimura & Imai 1967 and Yoshimura 1967) During perfusion with a K^+ free solution normal secretory potentials could be evoked for the first 15 min, thereafter they disappeared The secretory rate was severely reduced during perfusion with a K^+ free solution in accordance with results previously reported by Lundberg (1957c) Another important finding was the existence of normal secretory potentials during perfusion with a Cl free sulphate Ringer s solution.

This finding, which speaks against the hypothesis of Lundberg, is not very well documented. There is no quantitative treatment of the results only one typical secretory potential evoked during a period of perfusion with a sulphate Ringer s solution is shown. This only secretory potential (Fig. 3 in Imai (1965b) or Fig. 3 in Yoshimura & Imai (1967)) is, as was also stated by Burgen (see p 99 in Schneyer & Schneyer (1967)) remarkably small and of a somewhat irregular shape.

In accordance with the hypothesis of Lundberg, Imai denied that secretory potentials could be evoked during perfusion with a Cl^- free nitrate Ringer s solution. However Lundberg (1957c) was able to evoke small secretory potentials during this condition. This discrepancy may be explained by the fact that Imai used electrical stimulation of the parasympathetic nerves to the gland in these experiments whereas Lundberg used close intraarterial injection of ACh since he had found that during perfusion with a nitrate Ringer s solution the ganglia between the pre- and post-synaptic

parasympathetic nerves did not function properly (These ganglia are situated within the salivary glands and it is therefore extremely difficult to stimulate directly the postsynaptic nerves)

Imai concluded that the secretory potential could not readily be explained by assuming the existence of an active Cl transport. He suggested that ACh acted by increasing the permeability to K^+ This would cause an *increase in membrane potential since the membrane potential would tend* to approach E_K Imai (1965b) finally remarked Thus the secretory potential is not the causal factor of salivary secretion but is a phenomenon which often appears concomitant with the secretion Imai however never was able to argue against the results of the important experiments of Lundberg in which it was shown that the size of the secretory potential was independent of the level of the resting membrane potential Thus Imai presented an alternative and attractive hypothesis explaining the mechanism of the secretory potential, but was unable to remove the solid foundation of the Lundberg hypothesis.

The author's own studies on the cat submandibular gland.

In some previous papers (Petersen & Poulsen 1966 1967c) findings were reported that were in accordance with the hypothesis of Lundberg. It was shown that some inhibitors of active transport (acetazolamide, strophanthin-G cyanide and 2,4-dinitrophenol) reduced the size of the secretory potentials and the secretory rate to about the same degree and with about the same time course The important finding of Imai that augmentation of the extracellular K^+ concentration reduced the amplitude of the secretory potential was, however also confirmed. This was explained within the framework of the Lundberg hypothesis (Petersen & Poulsen 1967b) Since augmentation of the extracellular K^+ concentration inhibited the secretory rate and the secretory potential to the same degree this might be due to an inhibitory effect of the high extracellular K^+ concentration on the Cl pump being responsible for both the establishment of the secretory potential and the formation of saliva.

Douglas & Poisner (1963) had shown that salivary secretion depended on the presence of extracellular Ca^{2+} It was therefore decided to investigate what would happen to the secretory potential after removal of Ca^{2+}

A condition has never been described, in which salivary secretion occurs without the establishment of secretory potentials, the reverse situation can, however easily be created

from the perfusion fluid (A) The surprising result was that the size of the secretory potential was not reduced during perfusion with a Ca^2 free solution in spite of a severely reduced secretory ability (Fig. 2 in A) This result would seem to argue against the hypothesis of Lundberg. However it was decided, again, to discuss the result within the framework of the Lundberg hypothesis. *One of the possible solutions, to explain the apparent discrepancy* between the effect on the secretion and the secretory potential of reducing the extracellular Ca^2 concentration, mentioned in the discussion of A, deserves a comment. The effect of lowering the extracellular Ca^2 concentration might be to increase the water permeability in the duct system If this were true the reabsorption of NaCl always occurring at this site would result in an enhanced reabsorption of water It was therefore conceivable that in spite of an uninhibited primary secretory process in the acinus the secretory rate could be inhibited because of the enhanced reabsorption of saliva in the duct system occurring during the period of perfusion with a Ca^2 free fluid. The answer was sought by injecting retrogradely into the duct system THO and measuring the appearance of tracer in the perfusion fluid leaving the gland vein When comparing the rate of appearance of tracer in the perfusion fluid during control conditions and during perfusion with a Ca^2 free fluid no apparent difference could be seen. It was therefore concluded that the water permeability in the duct system was not markedly changed after removal of Ca^2 from the perfusion fluid. However this conclusion is only justified if the transfer of THO from the duct lumen to the perfusion fluid was not limited by the flow of perfusion fluid through the gland. The dissociation described in A between the secretory process and the secretory potential was not regarded as a challenge to the hypothesis of Lundberg.

Imai had described the existence of normal secretory potentials during perfusion with a Cl free Ringer's solution, although his finding was insufficiently documented (see p. 17) Petersen & Poulsen (1968b) were able to provide the desired documentation. Also it was possible to show that after replacement of extracellular Cl by NO_3 relatively normal secretory potentials could be evoked (Petersen & Poulsen 1969) However the result of these experiments can hardly be regarded as a disproof of Lundberg's hypothesis. The alleged Cl pump could conceivably be an anion pump and therefore transport nitrate as well as sulphate. This would of course not explain why salivary secretion was severely reduced when Cl was replaced by sulphate or nitrate, but other things, for example a low sulphate permeability at the luminal membrane, might explain that. A better experiment, therefore, had to be done. The essential question was, whether the contraluminal acinar cell membrane could generate a normal secretory po-

tential when the anion concentration in the extracellular fluid was very low In the experiments reported in F the submandibular gland was per fused with an isotonic solution containing (mM) sucrose 269 K_2SO_4 2 $MgSO_4$ 1 Na_2HPO_4 2.4 NaH_2PO_4 0 6 and glucose 5.5 The sucrose solu tion thus contained 11 4 meq/l anion whereas normally 150 meq/l anion is present. ACh evoked normal secretory potentials during perfusion with this low anion solution (Fig. 1 in F) The difference between the mean value of the sizes of the secretory potentials recorded in control and sucrose solution respectively was not significant.

The results obtained in F seemed to favour the hypothesis of Imai However the fact that Lundberg (1957b) had been unable to influence the secretory potential markedly by large variations in the resting membrane potential remained a serious problem. The results reported in B showed that the presence of the inhibitor 2 4-dinitrophenol (10^{-4} M) in the per fusion fluid did not inhibit an ACh-induced K^+ release at a time when both salivary secretion and active K^+ uptake following the release were severely reduced. According to Imai K^+ release and secretory potential were two signs of the same phenomenon and it should therefore be possible to show that DNP did not influence the size of the secretory potentials. However Petersen & Poulsen (1967c) had shown earlier that DNP (10^{-4} M) did indeed reduce the amplitude of the secretory potentials. In these experiments the glands were repeatedly stimulated during the period of perfusion with the DNP containing solution. Since the gland cells are unable to accumulate K^+ in this condition and lose K^+ upon each stimulation they must gradually have become K^+ depleted It was therefore important that the experiments were repeated, but in such a way that only a few ACh injections were given during one period of perfusion with the DNP containing solution. The results of those experiments are reported in C. It was, indeed shown that now the presence of DNP did not reduce the amplitude of the secretory potentials. The finding that the size of the secretory potentials were not reduced during perfusion with a DNP containing Locke s solution at a time when salivary secretion and K^+ uptake were nearly abolished did indicate that it was not necessary to assume the existence of an active elec trogenic pump mechanism in order to explain the secretory potential Now the case for the hypothesis of Imai seemed very strong.

According to this hypothesis variations in the extracellular K concentration should influence the magnitude of the hyperpolarization Imai (1965b) had shown that after augmentation of the perfusion fluid K^+ concentration from 4 to 10 meq/l the secretory potentials were nearly abolished. Qualitatively this was confirmed by Petersen & Poulsen (1967b) It seemed likely that the size of secretory potentials evoked at subnormal

extracellular K^+ concentrations should be enhanced as compared to control levels. During perfusion with a K^+ free solution Imai (1965b) was unable to show this. In the first 20 min after introduction of the K^+ free solution the secretory potentials were of normal size, thereafter they disappeared. The dependence of the secretory potentials on the perfusion fluid K^+ concentration was reinvestigated in D. The resting membrane potential did not depend markedly on the K^+ concentration in the perfusion fluid. During perfusion with the K^+ free solution the resting potentials were slightly enhanced and at a K^+ concentration of 20 mM they were slightly reduced. However the size of the secretory potential was clearly dependent on the extracellular K^+ concentration. During perfusion with the K^+ free solutions the amplitude of the secretory potentials was significantly larger than in the control periods and at an extracellular K^+ concentration of 20 mM the size was only half of that seen in the control periods (Fig. 3 in D).

We have now 2 important pieces of information. Burgen (1956) showed that ACh releases K^+ from the gland cells and in D it has been shown that the ACh-induced hyperpolarization is highly dependent on the extracellular K^+ concentration. We may now ask to which other ion movement the K^+-efflux is coupled. One possibility is that the K^+ efflux is accompanied by Cl^- efflux another one that Na^+ influx occurs concomitantly with the K^+ efflux. Finally a combination of these processes might occur. When one compares the intracellular concentrations of Na^+, K^+ and Cl^- calculated on the basis of determinations of the total content of these ions in homogenates of the dog submandibular gland during resting conditions with that during maximal stimulation it is apparent that K^+ lost during stimulation is being replaced by Na^+ (Imai 1965b). In B it was shown that after replacement of extracellular Na^+ by tetraethylammonium (TEA^+) the stimulation-induced K^+ release was severely diminished (Fig. 6 in B). This might indicate that K^+ efflux only occurs during conditions where Na^+ influx (or cation influx) is possible. If extracellular Na^+ is replaced by Li^+ there is primarily no inhibition of the stimulation induced K^+ release (Fig. 5 in B). After replacement of the perfusion fluid Na^+ by TEA the amplitude of the secretory potentials was significantly enhanced. This finding is in agreement with the hypothesis mentioned above that normally *i.e.* when the extracellular Na^+ concentration is high a Na^+ influx tending to reduce the membrane potential occur after stimulation. The level of the resting membrane potential was hardly affected after replacement of Na^+ by TEA. In F it was shown that the maximal level of the membrane potential during ACh stimulation was higher during perfusion with a low Na^+ sucrose solution than during control conditions. Finally some results reported in D show that the dependence of the amplitude of the secretory potential on

the extracellular K^+ concentration is much more marked during perfusion with low Na TEA solutions than normally (Fig. 6 in D) All these pieces of evidence are most easily explained by assuming that the mechanism of action of ACh on the contraluminal acinar cell membrane is to increase the conductances for K^+ and Na

It would be important to have some quantitative information about the strength of these currents. It is probably a reasonable assumption (as will be discussed in the following chapter) that both the secretory potential and the K^+ release are two signs of the same basic phenomenon the increased membrane permeability to K^+ and Na after stimulation. The initial K^+ efflux expressed per area of contraluminal cell membrane is about 40 nmol/cm^2/sec in the dog submandibular gland (Burgen 1956) but about 400 nmol/cm^2/sec in the cat sublingual gland (Lundberg 1958) The difference between these two figures is probably largely due to the difficulties in estimating the area of contraluminal cell membrane per g gland weight (Lundberg 1958) According to Lundberg this K^+ current amounts only to 1/50-1/100 of the transfer of charge needed for the establishment of the secretory potential This calculation is, of course, based on the very low values for the specific membrane resistance that Lundberg (1957b) arrived at. As already mentioned (p 16) it is unlikely that these low values are true. In another type of cell membrane, that has been more thoroughly investigated namely the squid axon, where a permeability increase also causes a change in membrane potential the maximal potential change during an action potential is about 100 mV At the time of maximal current density there is a net inward current of 1.2 mA/cm^2 (Hodgkin & Katz 1949) The above calculated K^+efflux is, expressed in these units about 4-40 mA/cm^2 However in the case of the acinar cell membrane the change in potential is only about 30 mV Furthermore the time resolution for the method on which the determination of the K^+ efflux rests is very poor (cf p. 27) so that it is probable that the value of 4-40 mA/cm^2 does not represent the maximal K^+ current density It is therefore likely that the K^+ efflux must be accompanied by a cation influx of about the same magnitude, probably dominated by Na Other cations (e g Ca^{2+}) might, however make minor contributions. At present it is impossible to know whether the effect of ACh is to increase generally the membrane permeability to ions or whether it is only a selective permeability increase for Na and K^+

It is apparent that the author in the early stages of his investigations favoured the electrogenic pump theory whereas it is now thought more likely that a permeability increase can explain the secretory potential. A similar development has been seen in another section of physiology Kehoe

& Ascher (1970) describe how it was previously thought that an electrogenic pump could explain the hyperpolarization seen in the abdominal ganglion of *Aplysia*. Now it is obvious that it is caused by an increase in K^+ permeability. The errors underlying the former interpretation, summarized in the paper of Kehoe & Ascher (1970) shall be briefly mentioned here since they may be relevant in understanding how the mechanism underlying the secretory potential was misunderstood. Results of two types of experiments are usually present when it is suggested that a hyperpolarization is caused by an electrogenic ion pump. 1) Experiments in which it is shown that the post-synaptic potential cannot be reversed by even large changes in the resting membrane potential (e.g. Lundberg 1957b). 2) Experiments in which it is shown that metabolic inhibitors (e.g. DNP, cyanide or strophanthin-G) inhibit the hyperpolarization (e.g. Petersen & Poulsen 1967c). Commenting on the first type of experiments Kehoe & Ascher explain that a relatively large distance between the site of the tip of the micro-electrode and the site of the permeability increase can give discrepancies between the equilibrium and the reversal potential. Also it has been shown that after application of γ-aminobutyric acid (GABA) to the dendrites of Mauthner neurones the conductance change can only be appreciated up to a few tenths of a micron from the site of application although there is hardly any decrement in the potential change over the same distance. The difficulty with the other type of experiment is, as has also been discussed in C, that metabolic inhibitors through inhibition of the Na-K^+ pump alter the electrochemical gradients across the cell membrane for at least these 2 ions.

Lundberg (1958) in his review "Electrophysiology of salivary glands" writes: "It is suggested that the secretory potentials should not be called post-synaptic potentials. The term postsynaptic potential should be reserved for such electrical changes as represent an immediate link between the attachment of the transmitter substance and events leading up to activity or inhibition of activity in the postsynaptic cell, or to potentials which have this origin even if the original function of the cell has become vestigial." It is felt, however, by the author that these secretory potentials should be included in the group of post-synaptic potentials. The processes occurring in the synapse between the post-ganglionic parasympathetic nerve cells and the acinar cells are not in principle different from what occurs in the neuromuscular transmission. At both sites ACh increases the permeability to ions in the post-synaptic membrane, which leads to a change in membrane potential.

The stimulation induced potassium release from the gland cells to the blood side

Burgen in his now classical paper from 1956 described a number of important experiments concerning the transport of K^+ in the salivary glands. The aim of the paper was to describe in greater detail than had previously been done the excretion of K^+ into the saliva. Burgen was able to show that there were essentially two phases in the secretion of K^+ into the saliva. The first phase after start of stimulation was a short lasting very marked secretion of K^+ most of the K^+ secreted coming from the gland cells. In the following phase the secretion of K^+ was more modest and the K^+ secreted came from the plasma but was transported through the gland cells. Therefore in the first phase the intracellular K^+ concentration must be declining. Furthermore it was shown that concomitantly with the secretion of K^+ into the saliva during phase 1 there was a secretion of K^+ into the blood with the same time course. In experiments where different stimulation intensities were employed it was apparent that there existed a proportionality between the K^+ release into the saliva and the blood. Burgen therefore suggested that the mechanism under lying the K^+ transfer from gland cell to saliva and from gland cell to blood could not be different.

Burgen described this phenomenon in three salivary glands, the dog submandibular and parotid glands, and the cat submandibular gland. Thaysen et al (1954) had already shown that the K^+ concentration in the first salivary samples, from the human parotid gland obtained after start of stimulation were higher than in those taken after a longer time of stimulation had elapsed. Also in the human lacrymal gland Thaysen & Thorn (1954) described the existense of an extra K^+ release to the tear side following an increase in secretory rate. A similar phenomenon was described by Öbrink & Waller (1966) in the gastric juice A K^+ release to the blood side after start of secretin-induced secretion has recently been demonstrated in the cat exocrine pancreas (Case Harper & Scratcherd 1969) It may therefore be a reasonable assumption that all exocrine glands being able suddenly to change their level of activity will display a K^+ release to both the secretory side and the blood side when they are stimulated to secrete after a longer resting period Bur

gen's experiments only dealt with net movements and it was therefore in principle impossible to know whether the increased net transport of K^+ to the blood side after stimulation should be explained by diminished influx to the cells or an increased efflux. Schneyer (196 showed that the efflux of $^{42}K^+$ from prelabelled rat submandibular gland slices to the incubation medium was significantly enhanced during the presence of ACh This would seem to indicate that ACh increased K^+ efflux from the gland cells.

There has been some speculation about the possible mechanism behind the stimulation-induced K^+ release Burgen in his 1956 paper wrote It seems probable that the potassium influx into the gland is an active process with a high temperature coefficient, whereas the efflux of potassium is passive and has a relatively low temperature coefficient However later in his review in the Handbook of Physiology (Burgen 1967) (p 575) Burgen had changed his mind. It is therefore difficult to resist the idea that there must be an active transport of potassium out of the cell base perhaps coupled to chloride and sodium entry "Therefore in the beginning of this last quotation refers to the fact that stimulation of the gland causes an increase in the acinar membrane potential (secretory potential) which would of course tend to reduce a possible diffusion of K^+ out of the gland cells. The argument is understandable since at that time it was generally accepted that the secretory potential was caused by an active Cl transport. However if as has indeed been concluded in chapter 1 it is the diffusion of K^+ out of the cells that increases the membrane potential the argument becomes non valid. A third explanation for the K^+ release was given by Lundberg (1958) "It has been suggested (Lundberg 1957b) that an elevated hydrostatic pressure in the cell is of importance for the passage of secretion from the cell interior into the lumen over the inner cell membrane. But these factors will work also over the outer membrane, where the increased intracellular hydrostatic pressure would cause an outward filtration of water and such osmotically active substances as are available in the cell interior and permeable through the outer membrane for example potassium and sodium, with presumably chloride as anion. Since the intracellular concentration of potassium exceeds that of sodium it is not surprising that an outflux of potassium does occur" According to this hypothesis the K^+ release should be secondary to the secretory process proper and one would predict that during conditions where the ability of the gland to produce saliva was reduced there would be an inhibition of the K^+ release

From previous studies it was impossible to conclude anything about the mechanism underlying the stimulation-induced K^+ release. This therefore was the aim of B. In B only the K^+ release to the blood side was considered since only here it was possible to investigate the effect of alterations in the ionic composition of the extracellular fluid on the K^+ transport. Burgen had

shown that electrical stimulation of the parasympathetic nerves to the gland caused a K^+ release. In B it was shown that a K^+ release occurred following intra-arterial injection of both ACh and adrenaline. In the cat submandibular gland sympathetic stimulation normally causes secretion in contrast to what happens in some other glands (e.g. the cat parotid) and secretory potentials can be evoked by both electrical stimulation of the sympathetic nerves and intra-arterial injection of adrenaline (Lundberg 1955) So this is another example of the in other places rarely occurring synergism between the sympathetic and the parasympathetic nervous system. In the salivary glands both these nerves influence secretion membrane potential and K^+ release in qualitatively the same way This also seems to be true for the action on the salivary myoepithelial cells (Emmelin Garrett & Ohlin 1968 1969) Recently Young & Martin (1971) have shown that the composition of the primary secretion is the same after both sympatho- or parasympathomimetic stimulation.

One of the most important results described in B was, as has already been mentioned in chapter I that DNP (10^{-4} M) does not primarily influence the stimulation-induced K^+ release although the uptake of K^+ and the secretion were severely reduced (Fig. 3 in B) Similar results were achieved by using strophanthin-G although the inhibition of the secretory rate was not always marked in these experiments. It was concluded that the K^+ release was caused by an increased permeability to K^+ One would predict that the K^+ release should depend on the extracellular K^+ concentration in much the same way as was shown for the secretory potential in D Unfortunately such a series of experiments was not carried out. Some experiments in which the effect of perfusing with a K^+ free solution was tested were done. 10 min after introduction of a K^+ free solution ACh stimulation gave a K^+ release of the same magnitude as that seen in the control periods. However during the 10 min of perfusion with the K^+-free solution the gland had lost substantial amounts of K^+ which may explain why the K^+ release was not augmented. In the control period succeeding the period of perfusion with the K^+-free solution a very marked K^+ accumulation occurred and ACh stimulation 10 min after the reintroduction of the control solution resulted in a K^+ release markedly larger than in the control period preceding the period of the K^+ free perfusion. The interpretation of this is Normally not all intracellular K^+ is in a freely diffusible state. Following a period of K^+ uptake the freely diffusible K^+ pool seems to be temporarily increased

A number of experimentel situations in which salivary secretion was severely reduced whereas the ACh-induced K^+ release remained unaffected were established in B This was observed during perfusion with a Na free Li^+ solution, a Cl free nitrate solution and a DNP containing Locke s solution.

These results argue against the hypothesis of Lundberg. It is therefore reasonable to assume that the stimulation-induced K^+ release is caused by an increased permeability to K^+ In chapter I it was suggested that the K^+ release was coupled to Na uptake.

The reader has probably gained the impression that the secretory potential is caused by the permeability increase that causes the K^+ release. This is probably true although at present it is difficult to prove. First one may ask whether these two phenomena occur synchronously The acinar cell membrane potential increases about 1 sec after the intra-arterial injection of ACh has been given. With respect to the K^+ release it is only known that the K^+ concentration in the effluent from the gland has reached its maximal value in the first 20 sec collection period following the injection of ACh Secondly one may ask whether these two phenomena occur in the same cell types. The secretory potential (the type I potential) is derived from the acinar cells. It seems very likely that the K^+ release is also largely derived from the acinar cells, but other cell types may also contribute (B G) At present it seems reasonable as a working hypothesis to assume that the secretory potential and the K^+-release are two signs of the same basic phenomenon, namely the increased permeability of the acinar cell membrane induced by ACh

III

Stimulus-secretion coupling.

The primary secretory process.

A primary secretion with a composition with respect to osmolarity and concentration of Na K^+ and Cl close to that of a plasma *ultrafiltrate* is formed in the salivary gland acini (Martinez Holzgreve & Frick 1966 Young & Schögel 1966 and Mangos, Braun & Hamann 1966) Yoshimura, Inoue Imai & Yoshimura (1962) reported that the osmolarity and the Na concentration was smaller and the K^+ concentration somewhat higher in the dog submandibular primary secretion than in a plasma ultrafiltrate Recently it was shown by Martin & Young (1971) that the primary secretion in the rat sublingual gland, in contrast to previous micropuncture data from the rat submandibular and parotid glands, had a somewhat lower Na and a somewhat higher K^+ concentration than that of a plasma ultrafiltrate but the same osmolarity It would therefore appear that an *isotonic fluid* transport occurs in the salivary gland acini. Such an isotonic transport has been studied in some details in the proximal kidney tubule (Ullrich 1965) the gall-bladder (Diamond 1962a) and the intestine (Curran 1960) In the three last-mentioned tissues the fluid transport occurs without any appreciable trans-epithelial electrical potential difference (Frömter & Hegel 1966 Diamond 1962b and Curran 1960) However Machen & Diamond (1969) have recently shown that a very small potential difference across the gallbladder epithelium exists during maximal transport. In the salivary gland it is difficult to decide whether the fluid transport is electroneutral or not. In the submandibular gland it has not yet proved feasible to insert a micro-electrode into the acinar lumen. In the sublingual gland Lundberg (1957a) succeded in doing this. During maximal fluid transport there is a potential difference between lumen and the *interstitial fluid* of about 10 to 20 mV (*lumen negative with respect* to the interstitial flu d) This luminal negativity is due to the existence of the secretory potential and we know now that this is not directly related to the fluid transport, since it is possible to obtain normal secretory potentials during a number of experimentel conditions (during perfusion with a Cl free sulphate solution (Petersen & Poulsen 1968b) during perfusion with a Ca^2

free solution (A) and during perfusion with a NaCl free sucrose solution (F)) in which salivary secretion is abolished. It is possible therefore that the isotonic fluid transport occurring in the acini is in itself an electro-neutral process.

In recent years it has become apparent that there exist two types of salt transporting epithelia. One type transporting Na against a large electrochemical gradient without much water transport *e.g.* the frog skin (Ussing 1960) the toad bladder (Leaf 1965) and the main excretory duct in many salivary glands (Young, Schögel, Frömter & Hamann 1967 Martinez 1969 Knauf & Frömter 1970b c). In this type of epithelium it is possible that one pump extruding Na from the cells to the extracellular fluid partly or completely in exchange for K^+ can explain the main characteristics (Kofoed Johnsen & Ussing 1958 and Knauf & Frömter 1970b) In the other type of epithelium an isotonic water transport occurs, *e.g.* the gallbladder (Diamond 1962a) the proximal kidney tubule (Ullrich 1965) and the intestine (Curran 1960) In *this type of epithelium it is probable that we have to deal with two kinds of*

It is not easily understandable how hyperpolarization of the contraluminal cell membrane l ads to a negativity of the lumen with respect to the intest tal fluid The outwardly directed K^+ current across the contraluminal cell membrane could increase the intracellular negativ ty This could attract cations f om the luminal compartment as indicated in Fig. 1 This would give rise to current loops as indicated on Fig 1 If the potential d fference between the free surface of the sublingual gland and the h lus is measured one obtains an increased negativity of the hilus electrode after starting stimulation (Lundberg 1957a) As already described in chapter II a K^+ release across both nonjunctional cell membranes occurs after stimulation. As has also been discussed already it is l kely that the K^+ lease to the blood side is accompanied by Na uptake. There are some results to indicate that the same also applies t the luminal membrane. Petersen & Poulsen (1968a) showed th t the salivary Na concentr tion fter start of timulation was increased as compared to the t ady state level The *duration of the period in which there is an xcess salivary Na concentration is the* same as the duration of the period n which the gland cells tak up K^+ from the blood side and the salivary side. From Fig. 2,3 and 4 i Petersen & Poulsen (1968a) it is apparent, howeve that i the very f rst samples obtained fter start of stimulat on the Na concentr tion is relatively low Th might indicate that initially a N flu occurs through the luminal membrane. I the cat submandibular gland ther is, in contrast to what was observed for the cat sublingual gland, an itial positi ty of the hilus electrode with respect t the surface lectrode (Lundberg 1955) This means that the direction of th current loops indicated on F g. 1 would have t be reversed in the case of the cat submandibular gland This would indicate that the luminal cell mem brane also hyperpolarizes ft stimulation. As mentioned in D t is possible that ACh can evoke Na and K^+ urrents across the ac nar ell membranes and that the mutual relationship between the trengths of these current can ary in different salivary gland membranes. In the submandibular gland t would appear that K^+ ffl dominates over Na nflu t both contraluminal and luminal cell membrane In the subl ngual gland t would appear that K^+ fflu dominates t th ontraluminal cell membrane whe as Na nflu dominates at the luminal membrane

pumps. A normal Na K$^+$ pump which has the usual task of maintaining a low intracellular Na concentration and a high intracellular K$^+$ concentration and another pump directly responsible for the isotonic fluid transport (Maude 1969 1970, Whittembury & Fishmann 1969 and Whittembury & Proverbio 1970) this might be an electroneutral NaCl pump (Diamond 1962b) or an electrogenic Na pump (Whittembury 1971) but other possibilities as *e.g.* a volume pump (Frederiksen & Leyssac 1969) also exist. It seems most likely that the salivary gland acini belong to this second type of epithelium.

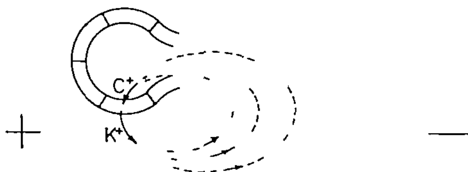


Fig. 1 A schematic illustration to explain the increased luminal negativity caused by the hyperpolarization of the contraluminal aciner cell membrane. C$^+$ denotes cation.

Hoffman & Kregenow (1966) first suggested the existence of two different kinds of Na pumps. They showed that two components of the Na efflux from erythrocytes could be distinguished by means of the agents strophanthin-G and ethacrynic acid. Whittembury & Fishman (1969) Whittembury & Proverbio (1970) and Proverbio Robinson & Whittembury (1970) have since shown that the Na extrusion from kidney tubules has two components. One part of the Na is extruded together with Cl this Na efflux can be inhibited by ethacrynic acid another part is exchanged for extracellular K$^+$ this Na extrusion and K$^+$ uptake can be inhibited by strophanthin-G.

In Fig. 3 in B it is seen that strophanthin-G (10^{-4} M) severely inhibited K$^+$ uptake following a stimulation-induced K$^+$ release whereas the secretory rate was hardly affected. This finding, which was not discussed in B caused after appreciation of the findings of Whittembury a further investigation into the effects of ethacrynic acid and strophanthin-G on K$^+$ transport and secretion, the results of which are reported in G It was again shown that it is possible to abolish K$^+$ uptake by strophanthin-G without inhibiting the salivary secretion rate (Fig. 1 in G) On the other hand ethacrynic acid could inhibit severely

the secretory rate and hardly affect the K^+ uptake (Fig. 2 in G) This might indicate the existence of at least 2 different active Na transport mechanisms. One, probably a Na K^+ exchange pump sensitive to strophanthin-G and the other a pump responsible for transport of salt and water into the acinar lumen, sensitive to ethacrynic acid

Unfortunately the salivary gland acinus is a somewhat more complex epithelium than several of the other isotonic fluid transporting tissues already mentioned. Besides the mechanisms responsible for the fluid transport proper there must also exist a mechanism concerned with the activation of the transport, since the salivary glands are not working continuously as e.g. the gallbladder or the proximal kidney tubule. When studying the effect of varying the ionic composition of the perfusion fluid on the rate of the ACh induced salivary formation we must expect an influence on both the activation and the transport processes, impeding a precise analysis of these processes.

The importance of the ACh-induced ionic currents for the secretory process.

Douglas & Poisner (1963) showed that the ability of the cat submandibular gland to secrete was severely reduced after a longer period of perfusion with a Ca^{2} free fluid In contrast to this Lundberg (1957c) reported that in the cat sublingual gland the secretory rate was not influenced after removal of Ca^{2} from the perfusion fluid. Douglas & Poisner (1963) also showed that a reduction of the perfusion fluid Ca^{2} concentration to 25% of the normal level reduced the secretory ability somewhat whereas an enhanced extracellular Ca^{2+} concentration only augmented the secretory rate when a submaximal ACh stimulus was used. Reintroduction of Ca^{2+}-containing solution to a salivary gland that had for some time been perfused with a Ca^{2+} free solution resulted in itself (i.e. without ACh stimulation) in a secretory response. Mc Carthy & Sheehan (1966) have later shown that this secretion occurring after reintroduction of Ca^{2+}-containing solution is due to stimulation of parasympathetic ganglia. In A it was shown that the amplitude of the secretory potentials was uninfluenced by even long periods of perfusion with a Ca^{2+} free solution in spite of an abolished secretory ability Results reported in G show that also the stimulation-induced K^+-release is independent of the presence of extracellular Ca^{2+} Therefore, reduction of the level of Ca^{2+} in the perfusion fluid does not inhibit salivary secretion by interfering with the ACh-induced permeability changes at the contraluminal acinar cell membrane and the subsequent movements of K^+ and Na The site of interaction of Ca^{2+} with the secretory process must be at a later stage in the chain of events that

eventually causes the formation of the primary secretion As discussed in A and G the role of Ca^{2+} might be a passive one namely to ensure normal permeability conditions especially intercellularly in the acinus. It would be more exciting though if ACh could influence Ca^{2+} transport in such a way as to increase the concentration of Ca^{2+} intracellularly which might then trigger off the secretory process. As discussed at some length in chapter I the ACh induced K^+ release seems to be accompanied by Na uptake. It cannot be excluded however that a small part of the inward current could be carried by Ca^{2+} Dreisbach (1964) showed the existence of an enhanced influx of $^{45}Ca^{2+}$ to slices of the rat parotid gland after stimulation with ACh Since ACh evokes a transcellular fluid movement which might in itself increase the uptake of extracellular ^{45}Ca, this finding may not be very important. The experiments should be repeated but in such a way as to ensure that no fluid transport occurred (e.g. in a Cl free sulphate Locke s solution) Still it is very difficult to do influx studies with a sufficiently high time resolution. Recently Nielsen & Petersen (1972) have shown that both ACh and adrenaline increase the efflux of ^{45}Ca from the prelabelled cat submandibular gland The effect was very marked. However it was not possible to detect an ACh evoked rapidly occurring ^{45}Ca influx concomitant with the enhanced Na and K^+ fluxes. Nielsen & Petersen (1972) concluded that the ACh and adrenaline induced ^{45}Ca efflux from the prelabelled cat submandibular gland was due to a release of Ca^{2+} from the organelles. Selinger Nalm & Lassor (1970) have shown that the salivary glands possess a powerful Ca^{2+} accumulating system in the microsomes apparently capable of reducing the Ca^{2+} concentration in the cytoplasm to about 0.5 μM The electrochemical gradient for Ca^{2+} across the contraluminal acinar cell membrane is of course very much in favour of Ca^{2+} uptake. If ACh did evoke an enhanced permeability to Ca^{2} this would cause an enhanced Ca^{2} uptake. In the adrenal medulla Douglas, Kanno & Sampson (1967b) were able to show that ACh evokes an increase in the permeability to both Na^+ and Ca^{2+} with a subsequent Na and Ca^{2+} influx. The cells of the adrenal medulla have some properties, *e.g.* a relatively low resting membrane potential in common with the salivary acinar cells, although ACh stimulation in the adrenal medulla causes depolarization rather than hyperpolarization (Douglas, Kano & Sampson 1967a)

The problem of stimulus-secretion coupling was attacked from a somewhat different angle in E. The results discussed in chapter I and II have shown that the action of ACh on the acinar cells is mainly to cause K^+ release and Na uptake. The aim of the experimental work reported in E was to test whether variations in the strengths of these ACh-evoked currents would influence the secretory rate The strengths of these currents are determined by the electrochemical gradients for K^+ and Na across the contraluminal cell

membrane and by the permeability. The electrochemical gradients were influenced by varying the K^+ and Na concentrations of the perfusion fluids used. In experiments in which the extracellular Na was reduced replacing NaCl by sucrose it was seen that the secretory rate (ACh-evoked) was very dependent on the perfusion fluid NaCl concentration. At an extracellular NaCl concentration of half the normal the secretory rate was halved as compared with control conditions (Fig. 2 in E). Lundberg (1957c) had shown previously that after replacement of 50% of the perfusion fluid Cl by NO_3 the secretory rate was hardly affected and Petersen & Poulsen (1969) showed that replacement of 50% of the perfusion fluid Cl by sulphate did not influence the secretory rate markedly. The experimental result in E must therefore have been caused by the reduced Na rather than by the reduced Cl^- concentration. This also coincides nicely with another experiment by Petersen & Poulsen (1969) in which it was shown that after replacing half the perfusion fluid Na by Li^+ the secretory rate was halved as compared with the control level. A simple hypothesis to account for these findings would be that the primary secretion was formed by a Na pump. This alleged pump might require a certain intracellular Na concentration to operate at a maximal rate. Alternatively the Na-influx or the thereby enhanced intracellular Na concentration could activate the solute pump through some other messenger (possibly Ca^{2+}).

In another experimental series the effect of varying the perfusion fluid K^+ concentration on the secretory rate was tested. During perfusion with a K^+ free solution the secretory rate was significantly enhanced above the rate seen during control conditions. On the other hand during perfusion with a solution containing 20 mM K^+ the secretory rate was only 50% of the control level (Fig. 3 in E). It may seem strange that removal of extracellular K^+ should improve the secretory ability of the gland. In both the proximal kidney tubule (Maude 1969) and the gallbladder (Frederiksen & Leyssac 1969) it has been shown that the transcellular fluid transport is severely reduced after removal of external K^+. The presence of an activator system being influenced favourably by the lowered extracellular K^+ concentration, in the salivary gland, may explain this discrepancy. Also the secretory rate in the above mentioned experiments was already measured 5 min after introduction of the K^+ free fluid, it is conceivable that the secretory rate would decline after a longer period of exposure to a K^+ free solution. The very short period of perfusion with K^+ free fluid employed in our experiments were chosen to minimize loss of intracellular K^+.

There is, however, also a discrepancy between these findings and those of Lundberg (1957c) on the cat sublingual gland. Lundberg reported the results of 4 experiments in which the salivary secretory rate was severely reduced

during perfusion with a K^+ free solution. In a typical experiment (Fig. 6 in Lundberg 1957c) it is seen that the volume of saliva secreted after ACh stimulation 5 min after introduction of the K^+ free fluid was only 25% of that secreted a few min after reintroduction of the control solution. Imai (1965b) (the dog submandibular gland) shows in a typical experiment (it seems that secretion was evoked via electrical stimulation of the lingual nerve) that the secretory rate was halved as compared to control conditions 5 min after introduction of K^+ free fluid. However the results in E are supported by previous findings of Petersen & Poulsen (1967b Fig. 4) showing that the secretory rate is enhanced after reducing the extracellular K^+ concentration to 0.5 or 0.2 mM.

Why should the salivary glands secrete at a higher rate under conditions where the electrochemical gradient favouring K^+ efflux was enhanced? We know that this would cause a higher membrane potential during ACh stimulations and this would cause a larger electrochemical gradient in favour of Na influx. The reduced extracellular K^+ concentration could therefore influence the secretory rate by augmenting Na influx during stimulation. Augmentation of the perfusion fluid K^+ concentration would tend to reduce the stimulated membrane potential thus reducing the electrochemical gradient in favour of Na influx and therefore, presumably the secretory rate.

One question remains to be answered. How can Na influx activate the secretory mechanism? An enhanced intracellular Na concentration would cause an enhanced activation of the Na K^+ activated ATP'ase presumably responsible for the extrusion of Na and uptake of K^+. Other enzymes related to salt transport might also be stimulated. Also an enhanced intracellular Na concentration could somehow cause a release of Ca^{2+} from the larger microsomal Ca^{2+} pool (Selinger Naim & Lasser 1970) or cause an enhanced uptake of Ca^{2+} from the extracellular fluid. It may be of interest in this connection to mention an interesting result of Baker Blaustein, Hodgkin & Steinhardt (1969) obtained from experiments on the squid axon and of Glitsch Reuter & Scholz (1970) in auricles from the guinea-pig. They showed that Ca^{2+} influx is dependent on the intracellular Na concentration in such a way that e.g. the Ca^{2} influx in the auricles was doubled after augmentation of the intracellular Na concentration from 10 meq/l to about 35 meq/l. Stimulation of the salivary glands causes an enhanced Na uptake. Injection of 10 µg ACh to a cat submandibular gland causes a release of about 10-20 µeq K^+ from the cells to the perfusion fluid. If one assumes that the K^+ lost is replaced by Na (Burgen 1956 (table 1) and Imai 1965b (table 2)) the intracellular Na concentration which is relatively low during resting conditions (about 10 meq/l according to Burgen (1967) or 3 meq/l according to Imai (1965b)) would increase to about 10-20 meq/l (calculated on the assumption of an intracel

lular space of about 1 ml, which is reasonable since the weight of the cat submandibular gland is about 1 to 2 g) Thus the increase in intracellular Na concentration following ACh stimulation is of a magnitude that in other (excitable) tissues causes a markedly increased Ca^{2+} influx. If a similar mechanism was operational in the salivary glands the importance of the ACh induced Na influx could be due to its influence on Ca^{2+} influx.

Finally it should be mentioned that adenosine 3',5' monophosphate (cyclic AMP) could function as an intracellular activator of the secretory mechanism Berridge & Prince (1971) have shown that in the salivary glands of *Calliphora* cyclic AMP mimicks the action of 5-hydroxytryptamine (the genuine stimulator of secretion) Case Laundy & Scratcherd (1969) have demonstrated that dibutyryl-cyclic AMP (the dibutyryl derivative is assumed to penetrate cell membranes more easily) causes secretion in the perfused cat pancreas and that the hereby achieved secretory rate can be further augmented by adding theophylline to the perfusion fluid (theophylline inhibits the diesterase responsible for the breakdown of cyclic AMP)

Rasmussen (1970) has suggested that the primary action of a transmitter on an effector cell may be to cause influx of Ca^{2+} and to stimulate an adenyl cyclase. An increased level of the adenyl cyclase would create an increased intracellular level of cyclic AMP This might release Ca^{2+} from intracellular stores and thus furthermore increase the Ca^{2+} concentration in the cytoplasm. If cyclic AMP were a crucial factor in stimulus-secretion coupling one should be able to demonstrate an increased intracellular concentration of cyclic AMP after stimulation as compared to control levels. In the exocrine pancreas Johnson, Sherratt, Case & Scratcherd (1970) showed that the concentration of cyclic AMP was trebled 30 sec after an intravenous injection of secretin and reached a maximal level after 1 min The secretion started 45 sec after the injection. It is thus possible that cyclic AMP is intimately involved in stimulus-secretion coupling in the exocrine pancreas. Such experiments have not yet been undertaken in the salivary glands, and it must be admitted that the problem concerning the involvement of cyclic AMP in stimulus-secretion coupling is at present obscure.

IV

A model for the primary secretion process

A brief description of certain aspects of the ultrastructure of the acinar cells is needed at this stage As mentioned in G intercellular spaces exist in the salivary gland acini. These are long, narrow dead-end channels not dissimilar to those in the gall-bladder the function of which is to provide the structural basis for the coupling between solute and water transport (Diamond & Bossert 1967) These channels may be closed at the luminal or the contraluminal surface. As also mentioned in G both types of channels may exist in the salivary glands. In the cat submandibular gland which is predominantly a mucous gland, it is probable that the channels are closed towards the contraluminal surface (G)

There are some similarities between the gall-bladder epithelium and the acini Both epithelia carry out an isotonic fluid transport, both possess long, narrow intercellular channels and in both cases the transepithelial specific resistance is very low (16 Ωcm^2 in the sublingual gland (Lundberg 1957b) and 28 Ωcm^2 in the rabbit gall-bladder (Diamond et al 1971)) It is therefore tempting to assume that the localization of the pump responsible for the fluid transport in the acinar cells should be similar to that of its counterpart in the gall bladder that is at the lateral cell membranes. Therefore a transport of NaCl from the intracellular space to the intercellular space is supposed to occur (Fig. 2) As previously discussed (G) it is impossible to know whether this pump is an electroneutral one as envisaged by Diamond (1962b) or an electrogenic one as has been shown to be true for the necturus proximal kidney tubule (Whittembury 1971) According to the results described in G this pump must be the site of action of the inhibitory agent ethacrynic acid. It would appear that the intercellular channels in the acini provide the structural basis for the standing-gradient flow system postulated by Diamond & Bossert (1967) (Fig. 2) This would explain the isotonicity of the primary secretion

The other type of active transport occurring in the acini is the Na K$^+$ exchange also indicated in Fig. 2 at both the luminal and contraluminal cell membranes. These are the sites of action of strophanthin-G These pumps maintain concentration gradients for Na and K$^+$ across the cell membranes.

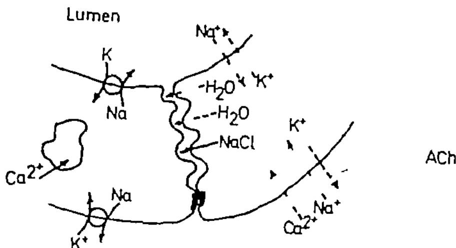


Fig. 2. A diagram showing the essential processes concerned with the formation of the primary secretion. The broken arrows represent passive ion transports, whereas the fully drawn arrows represent active (energy requiring) processes.

In the salivary glands this is an especially important task since stimulation of the glands causes release of K^+ and uptake of Na. As indicated in Fig. 2 it is possible that Ca^2 uptake occurs following stimulation. The mechanism underlying this uptake if it occurs at all is obscure, as is its significance. There is no doubt that a Ca^2 accumulating system is present in the microsomes (Selinger et al. 1970). This is also shown in Fig. 2. This figure illuminates our present state of ignorance. Although we can make reasonable assumptions as to the primary mechanism of action of ACh on the acinar cell membrane, we do not know how this initiates solute transport at the lateral membranes.

4) The reproducibility of the determinations of the K^+ concentrations and control experiments for possible Na K^+ interference during the flame photometry

Details about the methods employed are to be found in B and G All measurements were carried out in duplicate. 20 samples all containing the same perfusion fluid were analyzed for K^+ The mean value ± standard deviation was 4 10 meq/l ± 0 01 No precautions for the possibility of Na K^+ interference during the flame photometry were taken It is however most unlikely that variations in the perfusion fluid Na concentration should have influenced the results. Table III shows that even very large variations in Na concentration have very little influence on the measured K^+ concentration.

Table III

Measured K^+ concentrations (meq/l) in solutions with different Na concentrations but with a constant K^+ concentration (4 meq/l)

[Na] = 0	[Na] = 60	[Na] = 140
4 10	4 18	4 20
4 10	4 20	4 20
4 12	4 20	4 20
4 13	4 20	4 20
4 10	4.20	4 20
4 10	4 18	4 20
4 10	4 18	4 20
4 10	4 18	4 18

5) The magnitude of the secretory potentials

In A the mean value of the amplitude of the secretory potentials during perfusion with a control Locke's solution was about 17 mV (Fig. 3 in A) whereas later (C D and F) the mean value was always above ?0 mV (Fig. 3 in C) The method employed in A is described in detail by Petersen & Poulsen (1967b) The method employed in C, D and F is described in both C and D The only difference in method of possible significance is the nature of the micro-electrode impalement. In A a de Fonbrune micro-manipulator was used whereas in C D and F a stepping motor micromanipulator (Transvertex, Sweden) was employed It is possible that the stepping

motor device enables one to penetrate the cell membranes more gently than by using a manually operated micro-manipulator. Also the resting potentials are somewhat higher in C, D and F than in A. An alternative explanation for the higher values observed in the later studies is that a greater skill in the preparation of the gland had been achieved at that stage.

6) The reproducibility of the size of a drop of saliva.

In some of the original papers the secretory ability of a gland was assessed by counting the number of drops of saliva secreted after an injection of ACh. In one experiment 7 drops were weighed. The mean value ± standard error was 22.6 mg ± 0.5.

SUMMARY

The aim of the present work was to explain the mechanism underlying the hyperpolarization of the salivary gland acinar cell membrane and the potassium release from the cells after stimulation It was hoped thereby to learn more about the way in which the salivary secretory process is initiated.

Chapter 1

The secretory potentials that have been measured in different exocrine glands are described. Generally the potential change is slow and it is not an all or non phenomenon

Lundberg s studies into the mechanism underlying the secretory potential are reviewed Lundberg postulated that the secretory potential is the electrical sign of an active chloride transport being directly responsible for the formation of the acinar primary secretion.

Imai s studies into the mechanism underlying the secretory potential are subsequently reviewed Imai disagreed with Lundberg s hypothesis without disproving it. Imai suggested that the secretory potential is unrelated to the secretory process and is due to an ACh-induced membrane permeability increase to potassium.

Finally the authors own studies are mentioned. It was shown that an active chloride transport cannot be responsible for the establishment of the secretory potentials, since they are maintained during exposure of the gland to a chloride-free sucrose solution. It was also shown that the metabolic inhibitor 2.4 – dinitrophenol has no influence on the amplitude of the secretory potentials. This seems to indicate that the secretory potentials are caused by a permeability change at the cell membrane rather than by an active ion transport. It was finally shown that the magnitude of the secretory potential depends on the extracellular potassium concentration in such a way that the amplitude is enhanced after reducing the potassium concentration and reduced after augmentation of the potassium concentration. Replacement of extracellular sodium by tetraethylammonium causes an increased amplitude of the secretory potential and the dependence on the extracellular potassium concentration is more marked than normally The interpretation is The secretory potential is caused by an enhanced membrane permeability to potassium. This causes a potassium release. The outwardly directed potassium current is partly short-circuited by an inward sodium current.

Chapter II

A stimulation-induced potassium release has been described in a number of exocrine glands and it is suggested that this phenomenon is of importance in the transition from rest to activity occurring after stimulation of the salivary glands. The results of the author's own investigations show that potassium release is uninhibited by strophanthin-G and dinitrophenol indicating that it is a non-energy requiring process. Replacement of extracellular sodium by tetraethylammonium strongly inhibits the potassium release. This indicates that potassium release and sodium uptake are normally coupled. Finally it is discussed whether the secretory potential is the electrical sign of the ion movements described in this chapter

Chapter III

Our knowledge of the primary secretion process in the acini is first summarized and then compared with that of other tissues carrying out an isotonic fluid transport (the gall-bladder and the proximal kidney tubule)

The effect of strophanthin-G and ethacrynic acid on potassium transport is described. The conclusion is that the acinus possesses 2 types of sodium pumps. One is extruding sodium in exchange for potassium the other is directly concerned with the transport of solute into the acinar lumen.

The importance of calcium for the secretory process is described The possibility that an acetylcholine-induced calcium influx is of importance in the stimulus-secretion coupling is discussed.

Experiments designed to give information about the possible importance of the already mentioned acetylcholine-induced potassium and sodium currents are described. The secretory rate is very dependent on the extracellular sodium concentration even more than on the extracellular chloride concentration. Variations in the extracellular potassium concentration influence the secretory rate in such a way that augmentation causes an inhibition of secretion while the secretory rate is enhanced at a reduced potassium concentration. This is probably an indirect effect mediated by the imposed variations in stimulated acinar cell membrane potential influencing the rate of sodium influx Varying the parameters determining the rate of sodium influx during stimulation thus seems to influence the activation of the secretory machinery The ion movements causing the secretory potential are probably essential for the initiation of the secretion.

Chapter IV

On the basis of our knowledge of the acinar cell ultrastructure and by making comparison with the gall bladder epithelium a model for the transport events in the acini is suggested

RESUMÉ

Formålet med dette arbejde har været at søge at finde mekanismen ved den ændring i acinuscellemembranpotentialet og frigørelse af K^+ fra kirtelceller til ekstracellulærvædske der foregår når en spytkirtel stimuleres til at secernere. Det var håbet herigennem at belyse hvorledes spytsekretionen igangsættes.

Kapitel 1

Der gennemgås de indtil nu beskrevne sekretoriske potentialer i exocrine kirtler og visse fællestræk summeres op. Væsentligst af disse er at det drejer sig om langsomme potentialændringer og at de er graderede i størrelse.

Lundberg's studier over mekanismen ved det sekretoriske potential gennemgås. Lundberg placerede det sekretoriske potential meget centralt i sekretionsmekanismen idet han mente at det var det elektriske tegn på den aktive chloridtransport, der var ansvarlig for dannelsen af primærsekretet.

Imai's studier over mekanismen for etableringen af det sekretoriske potential gennemgås. Imai afviste dog på et ret løst grundlag Lundberg's hypothese og postulerede i stedet, at det sekretoriske potential intet havde med sekretionsmekanismen at gøre, men skyldtes en af acetylcholin fremkaldt øget permeabilitet for kalium i den acinære cellemembran med en heraf følgende udvandring af kaliumioner.

Forfatterens egne undersøgelser over det sekretoriske potential gennemgås. Først vises at det sekretoriske potential ikke kan skyldes en aktiv chloridtransport ind i cellerne, da det kan fremkaldes under perfusion med en chlorid-fri sukrose opløsning. Dernæst vises, at den metaboliske inhibitor 2,4-dinitrophenol ingen effekt har på størrelsen af det sekretoriske potential. Dette tyder på, at passive iontransporter kan forklare dette fænomen. Endelig vises at størrelsen af det sekretoriske potential afhænger af den ekstracellulære kaliumkoncentration således, at en øgning hæmmer potentialet, medens en sænkning øger størrelsen af det sekretoriske potential. Hvis perfusionsvædskens natrium erstattes med tetraethylammonium øges størrelsen af det sekretoriske potential og afhængigheden af den ekstracellulære kaliumkoncentration bliver tydeligere. Dette fund tydes således at mekanismen bag det sekretoriske potential er en øget membranpermeabilitet for kalium med en deraf følgende udvandring af kaliumioner. Denne

udadrettede kaliumstrom kortsluttes dog delvist af en indadrettet natrium strøm

Kapitel II

I en række exocrine kirtler er den stimulations-inducerede kaliumfrigørelse beskrevet, og det er således rimeligt, at antage at den har en eller anden betydning i omsætningen af kirtlens tilstand fra hvile til aktivitet. Forfatterens egne undersogelser viser at kaliumfrigørelsen primært er uafhængig af tilstedeværelsen af inhibitorerne dinitrophenol og g-Strophanthin, hvilket tyder på, at det drejer sig om en passiv proces. Hvis perfusionsvædskens natrium erstattes med tetraethylammonium hæmmes kaliumfrigørelsen stærkt. Dette tydes på den måde at kaliumfrigørelsen normalt finder sted samtidig med en natriumoptagelse. Til slut diskuteres hvorvidt det sekretoriske potential er et tegn på de i dette kapitel beskrevne ionvandringer

Kapitel III

Først omtales kort vor viden om den proces hvorved det acinære primærsekret dannes og der forsoges en sammenligning med andre organer der foretager en isoton vædsketransport som f eks. galdeblæren og den proksimale nyretubulus.

Dernæst gennemgås egne resultater af g-Strophanthins og ethacrynsyres indvirken på kaliumtransport og sekretion. Det konkluderes, at der i spytkirtelacini må findes 2 pumper der er beskæftigede med natriumtransport. En pumpe der transporterer natrium ud af og kalium ind i cellerne og en pumpe der sorger for transport af natriumchlorid og dermed vand ind i spytkirtellumen.

Derefter beskrives tidligere arbejder og egne undersogelser over calciums indflydelse i sekretionsprocessen. Muligheden for at et væsentligt element i stimulus-sekretionskoblingen er en af acetylcholin induceret øget indtransport af calcium i acinuscellen diskuteres.

I det følgende gennemgås egne undersogelser over hvorvidt de i de to tidligere kapitler beskrevne passive ionvandringer af kalium og natrium som startes af acetylcholinpåvirkning har nogen betydning for aktiveringen af kirtlen til at secernere Det vises, at sekretionshastigheden afhænger stærkt af den ekstracellulære natriumkoncentration en afhængighed der er meget mere udtalt end den tidligere af Lundberg beskrevne afhængighed af den ekstracellulære chloridkoncentration. Derudover vises at ændringer i den

ekstracellulære kaliumkoncentration influerer på sekretionshastigheden således at et fald i kaliumkoncentration øger sekretionshastigheden, medens en stigning hæmmer denne. Kaliumfundene kan fortolkes ved at antage at kaliumeffekten er indirekte, nemlig ved at påvirke natriumindvandringen via ændringer i det transmembranale potential. Det konkluderes at ændringer i de tilstande der er bestemmende for størrelsen af den passive indvandring af natriumioner ved stimulering af kirtlen, har betydning for aktiveringen af kirtlen. Det er således muligt, at de processer der ligger til grund for det sekretoriske potential har en væsentlig rolle at spille i aktiveringen af kirtlen til at secernere.

Kapitel IV

På grundlag af kendskab til acinuscellens ultrastruktur og ud fra analogislutning til specielt galdeblæreepithelet foreslås en model for hvorledes den isotone vædsketransport gennem acinuscellen kan tænkes at foregå.

ACKNOWLEDGEMENT

The present investigation was carried out in the years between 1966 and 1970 first at the Institute of Medical Physiology A and thereafter at the Institute of Medical Physiology C, University of Copenhagen after its establishment in 1967

The investigation was initiated following a suggestion by Professor dr med. N A. Thorn now head of the Institute of Medical Physiology C. Professor Thorn s unfailing interest in the project, his tremendous help in the initial stages and stimulating criticism throughout the period in which this work was carried out have been of the utmost importance for me

I am grateful to my friend Dr J Hedemark Poulsen for his contribution to the solution of the problems attacked in collaboration with professor Thorn in one of the previously published papers and also for many stimulating discussions thereafter

I want to thank professor med. dr Anders Lundberg (Gothenborg) for many important suggestions and for stimulating discussions at several occasions in Gothenborg.

Professor dr med. C. Crone gave valuable advice at some crucial stages in the first period of this investigation.

I want to thank professor dr med O Sten-Knudsen for helpful criticism of one of the original papers and this survey

It has been of great importance for the development of many of the ideas proposed and tested in this thesis that I have been able directly to meet and discuss with nearly all those actively engaged in research on glandular transport physiology I am particularly indepted to Dr J.A Young (Sydney) professor A.S V Burgen, F R.S (Cambridge) and professor J R. Martinez (San Salvador) for many fruitful discussions.

I want to thank Dr S Pors Nielsen for many interesting discussions on salivary gland Mg and Ca transport.

I am very grateful for the excellent help always given to me by Grete Lisa Pedersen. Grete Pedersen has brilliantly helped during the actual experiments and also typewritten all the manuscripts for the original papers and for this survey Miss Pedersen has also carried out the photography of the figures for the original papers produced by mrs. A. Blatt.

This investigation was financially supported by grants from Johann and Hanne Weiman s legacy The Danish State Research Foundation Carl and Ellen Hertz legacy The Foundation for the advancement of the medi

cal sciences, and Scandinavian Insulin Foundation. I am grateful for this generous financial support which has been essential for the accomplishment of the present investigation.

References

Baker P F M.P Blaustein A.L. Hodgkin & R.A Steinhardt, The influence of calcium on sodium efflux in squid axons. *J Physiol (Lond)* 1969 *200* 431-458. (34)

Bayliss, W M & J R. Bradford The electrical phenomena accompanying the process of secretion in the salivary glands of dog and cat. *Proc. Roy Soc. B.* 1886 *40*· 203-266 (11)

Berridge M J & W T Prince The electrical response of isolated salivary glands during stimulation with 5-hydroxytryptamine and cyclic AMP *Phil. Trans. Roy Soc. B.* 1971 *262* 111 120 (35)

Burgen A.S V The secretion of potassium in saliva. *J Physiol (Lond.)* 1956 *132* 20-39 (9 21 22, 24 25)

Burgen A.S V Secretory processes in salivary glands. In *Handbook of physiology* section 6 Alimentary canal, vol. II Secretion, 1967 pp 561 579 (9 25 34)

Burgen A.S V & N.G. Emmelin *Physiology of the salivary glands.* London Arnold. 1961 p 205 (Table I 12)

Case R.M., A.A. Harper & T Scratcherd, Water and electrolyte secretion by the pancreas. *In Exocrine Glands.* Eds Botelho, S Y., F.P Brooks & W B Shelley Philadelphia University of Pennsylvania Press. 1969 pp 39-56. (24)

Case, R.M T J Laundy & T Scratcherd Adenosine 3 5 -monophosphate (cyclic AMP) as the intracellular mediator of the action of secretin on the exocrine pancreas. *J Physiol (Lond.)* 1969 *204* 45-47P (35)

Coombs J.S., J C. Eccles & P Fatt, The electrical properties of the motoneurone membrane. *J Physiol. (Lond.)* 1955 *130* 291 325 (15)

Creed, K.E. & J A.F Wilson, The latency of response of secretory acinar cells to nerve stimulation in the submandibular gland of the cat. *Austr J Exp Biol. Med Sci.* 1969 *47* 135-144 (12)

Curran P F Na Cl and water transport by rat ileum in vitro. *J Gen. Physiol.* 1960 *43* 1137 1148 (28 29)

Dean, P M. & E K. Matthews, Miniature depolarisation potentials in pancreatic acinar cells. *J Physiol. (Lond)* 1968 *198* 90-91P (12)

Diamond J.M The reabsorptive function of the gall-bladder *J Physiol (Lond.)* 1962a, *161* 442-474 (28 29)

Diamond J M The mechanism of solute transport by the gall bladder *J Physiol. (Lond.)* 1962b *161* 474 502. (28 30 36)

Diamond J.M P.H Barry & E.M Wright, The route of transepithelial ion permeation in the gall-bladder In *Electrophysiology of Epithelial Cells*. ed. Giebisch, G. Stuttgart-New York Schattauer Verlag 1971 pp. 23-38. (36)

Diamond, J M & W.H Bossert, Standing-gradient osmotic flow *J Gen. Physiol 1967 50 2061 2083 (36)*

Douglas, W W., T Kanno & S R. Sampson Effects of acetylcholine and other medullary secretagogues and antagonists on the membrane potential of adrenal chromaffin cells An analysis employing techniques of tissue culture. *J Physiol. (Lond.)* 1967a, *188* 107 120. (32)

Douglas, W W T Kanno & S R. Sampson, Influence of the ionic environment on the membrane potential of adrenal chromaffin cells and the depolarizing effect of acetylcholine. *J Physiol. (Lond)* 1967b *191* 107 121 (32)

Douglas, W W & A.M. Poisner The influence of calcium on the secretory response of the submaxillary gland to acetylcholine or to adrenaline *J Physiol. (Lond.)* 1963 *165*. 528-541 (18 31 39)

Dreisbach, R.H., Calcium transfer in rat salivary and lacrimal glands. In *Salivary glands and their secretions*. Eds L.M. Screebny & I Meyer Oxford Pergamon Press. 1964 pp 237 249 (32)

Emmelin, N. Nervous control of salivary glands. In *Handbook of Physiology* Section 6 Alimentary canal vol II Secretion pp. 595-632, Washington American Physiological Society 1967 (9)

Emmelin, N J R. Garrett & P Ohlin Neural control of salivary myoepithelial cells. *J Physiol. (Lond)* 1968, *196* 381 396. (26)

Emmelin N J R. Garrett & P Ohlin, Motor nerves of salivary myoepithelial cells in dogs. *J Physiol. (Lond.)* 1969 *200*: 539-546. (26)

Frederiksen O & P P Leyssac, Transcellular transport of isosmotic volumes by the rabbit gall-bladder *in vitro*. *J Physiol. (Lond.)* 1969 *201* 201 224 (30 33)

Fritz, M.E. & S Y Botelho, Membrane potentials in unstimulated parotid gland of the cat. *Am. J Physiol.* 1969a, *216* 1180-1183 (Table II 13 17)

Fritz, M E. & S Y Botelho, Role of autonomic nerve impulses in secretion by the parotid gland of the cat. *Am. J Physiol.* 1969b *216* 1392 1398. (12)

Frömter E. & U Hegel Transtubuläre Potentialdifferenzen an proximalen

und distralen Tubuli der Rattenniere. *Pflugers Arch.* 1966 *291* 107 120. (28)

Glitsch H G H Reuter & H Scholz The effect of the internal sodium concentration on calcium fluxes in isolated guineapig auricles. *J Physiol (Lond.)* 1970 *209* 25-43 (34)

Graham, A.R. & G.W Stavraky Reversal of the effects of chorda tympani stimulation, and of acetylcholine and adrenaline as seen in the submaxillary salivary gland of the cat. *Rev Canad Biol* 1953 *11* 446-470 (39)

Henriques, B.L. & A.L. Sperling. Marking of sited cells after electrophysiological study *J Appl. Physiol* 1966 *21* 1247 1250 (Table II 13 17)

Hisado M & S Y Botelho, Membrane potentials of the in situ lacrimal gland in the cat. *Am. J Physiol.* 1968 *214* 1262 1267 (12)

Hodgkin, A.L. & B Katz, The effect of sodium ions on the electrical activity of the giant axon of the squid *J Physiol. (Lond.)* 1949 *108* 37 77 (22)

Hoffman J F & F.M Kregenow The characterization of new energy dependent cation transport processes in red blood cells. *Ann. N Y Acad. Sci.* 1966 *137* 566-576. (30)

Imai, Y Studies on the secretory mechanism of the submaxillary gland of the dog, part 1 Electrophysiological studies with microelectrode *J Physiol Soc. Japan* 1965a, *27* 304-312 (In Japanese) (Table II 12, 13 17)

Imai Y Study of the secretion mechanism of the submaxillary gland of dog, part 2. Effects of exchanging ions in the perfusate on salivary secretion and secretory potential with special reference to the ionic distribution in the gland tissue *J Physiol Soc. Japan* 1965b *27* 314-324 (In Japanese) (Table I 12 17 18 20 21 34)

Johnson M. H.S.A. Sherratt, R.M Case & T Scratcherd The effects of secretin, pancreozymin and acetylcholine on the concentration of adenosine 3 5-cyclic monophosphate in cat pancreas. *Biochemical J* 1970 *120* 8-9P (35)

Kehoe, J.S & P Ascher Re-evaluation of the synaptic activation of an electrogenic sodium pump. *Nature* 1970 *225* 820-823 (15 22 23)

Knauf H. & E. Fromter Die Kationenausscheidung der grossen Speicheldrusen des Menschen. *Pflugers Arch.* 1970a, *316* 213-237 (9)

Knauf H. & E. Fromter Elektrische Untersuchungen am Hauptausfüh-

rungsgang der Speicheldrusen des Menschen I Potentialmessung. *Pflugers Arch* 1970b *316* 238-258 (29)

Knauf H. & E. Frömter Elektrische Untersuchungen am Hauptausfüh rungsgang der Speicheldrüsen des Menschen II Bestimmung des Kurz schlussstromes. *Pflügers Arch.* 1970c, *316* 259 274 (29)

Kofoed-Johnsen V & H.H Ussing. The nature of the frog skin potential *Acta physiol scand.* 1958 *42* 298 308 (29)

Lassen, U V & O Sten Knudsen Direct measurement of membrane potential and membrane resistance of human red cells. *J Physiol (Lond.)* 1968 *195* 681-696. (13)

Leaf A. *Transepithelial transport and its hormonal control in the toad bladder Ergebn. Physiol.* 1965 *56* 216-263 (29)

Leeson C.R., Structure of salivary glands. In *Handbook of Physiology* sec tion 6 vol II pp 463-495 Washington Am Physiol Soc. 1967 (15)

Loewenstein, W R Permeability of membrane functions. *Ann. N Y Acad. Sci.* 1966 *137* 441-472. (16)

Lundberg, A., Secretory pot'ntials in the sublingual and submaxillary glands of the cat. *J Physiol. (Lond.)* 1954 *124* 25P (11)

Lundberg, A., The electrophysiology of the submaxillary gland of the cat. *Acta physiol. scand. 1955 35 1 25 (Table II 9 11 13 26 29)*

Lundberg, A. Secretory potentials in the sublingual gland of the cat. *Acta physiol. scand.* 1957a, *40* 21 34 (Table II 12, 13 14 28 29)

Lundberg, A. The mechanism of establishment of secretory potentials in sublingual gland cells. *Acta physiol. scand.* 1957b *40* 35-58 (15 16 20 22, 23 25 36)

Lundberg, A. Anionic dependence of secretion and secretory potentials in the perfused sublingual gland *Acta physiol. scand.* 1957c, *40* 101 112. (16 17 31 33 34)

Lundberg, A., Electrophysiology of salivary glands. *Physiol Rev* 1958 *38* 21 39 (12, 22, 23 25)

Machen T E & J M Diamond An estimate of the salt concentration in the lateral ntercellular spaces of 'abbit gall-bladder during maximal fluid transport. *J Membr Biol.* 1969 *1* 194-213 (28)

McCarthy D.M. & J D Sheehan The role of calcium ions in salivary secretion. *J Physiol. (Lond.)* 1966 *184* 81-82P (31)

Mangos, J A. G. Braun & K.F Hamann Micropuncture study of sodium and *potassium* excretion in the rat parotid saliva. *Pflügers Arch* 1966 *291* 99-106 (9 28)

Martin C.J & J A. Young. Electrolyte concentrations in primary and final

saliva of the rat sublingual gland studied by micropuncture and catheterization techniques. *Pflugers Arch.* 1971 *324* 344-360. (9 28)

Martinez, J R., Water and electrolyte secretion by submaxillary gland. In *Exocrine Glands.* Eds. Botelho, S Y F P Brooks & W B Shelley Philadelphia University of Pennsylvania Press. 1969 pp 20-30. (29)

Martinez, J R. H. Holzgreve & A. Frick, Micropuncture study of submaxillary glands of adult rats. *Pflügers Arch.* 1966 *290* 124-133 (9 28)

Maude, D L., Effects of K^+ and ouabain on fluid transport and cell Na in proximal tubule in vitro *Am. J Physiol.* 1969 *216* 1199-1206 (30,33)

Maude D L., Mechanism of salt transport and some permeability properties of rat proximal tubule. *Am. J Physiol.* 1970 *218* 1590-1595 (30)

Nielsen, S.P & O H Petersen Excretion of magnesium, calcium and inorganic phosphate by the cat submandibular gland *Pflügers Arch.* 1970 *318.* 63-77 (9)

Nielsen, S P & O H. Petersen Transport of calcium in the perfused submandibular gland of the cat. *J Physiol. (Lond)* 1972, *223* 685-697 (32)

Petersen O H. Some factors influencing stimulation-induced release of potassium from the cat submandibular gland to fluid perfused through the gland. *J Physiol. (Lond.)* 1970a, *208* 431-447 (13 20 21 25 26 27 30 38, 39 40)

Petersen, O H The effect of dinitrophenol on secretory potentials secretion and potassium accumulation in the perfused cat submandibular gland. *Acta physiol. scand.* 1970b *80* 117 121 (20, 23 38 40)

Petersen O.H The dependence of the transmembrane salivary secretory potential on the external potassium and sodium concentration. *J Physiol. (Lond.)* 1970c, *210* 205-215 (Table II 13 14 21 22 26 29 40)

Petersen, O H The importance of extracellular sodium and potassium for acetylcholine-evoked salivary secretion. *Experientia* 1970d,26 1103-1104 (32, 33 34 38)

Petersen O H., Secretory transmembrane potentials in acinar cells from the cat submandibular gland during perfusion with a chloride-free sucrose solution. *Pflugers Arch* 1971a, *323* 91 95 (14 20 29 40)

Petersen O H., Formation of saliva and potassium transport in the perfused cat submandibular gland. *J Physiol (Lond.)* 1971b *216* 129-142. (13 27 30 31 32 36 38 40)

Petersen O H. Initiation of salt and water transport in mammalian salivary

glands by acetylcholine *Phil Trans. Roy Soc B.* 1971c, *262* 307 314 (9)

Petersen O.H The ionic transports involved in the acetylcholine-induced change in membrane potential in acinar cells from salivary glands and their importance in the salivary secretion process. In *Electrophysiology of Epithelial Cells.* ed Giebisch G Stuttgart New York Schattauer Verlag. 1971d, pp. 207 224 (9)

Petersen, O H. & J H Poulsen, Inhibition of secretion and secretory potentials in the submandibular gland of the cat by acetazolamide *Experientia* 1966 *21* 821-823 (18)

Petersen O.H & J H. Poulsen Excretion of sodium and potassium in cat submandibular saliva. *Acta physiol. scand* 1967a, *70* 158 167 (9)

Petersen O H & J H. Poulsen, The effects of varying the extracellular potassium concentration on the secretory rate and on resting and secretory potentials in the perfused cat submandibular gland. *Acta physiol scand.* 1967b *70* 293-298. (18 20 34 40)

Petersen O H. & J.H. Poulsen Inhibition of salivary secretion and secretory potentials by g-strophanthin dinitrophenol and cyanide. *Acta physiol. scand.* 1967c, *71* 194-202. (18 20 23)

Petersen, O H. & J H. Poulsen, The secretion of sodium and potassium in cat submandibular saliva during the first period after start of stimulation. *Acta physiol. scand.* 1968a *73* 93-100 (29)

Petersen O.H & J.H Poulsen Secretory potentials, potassium transport and secretion in the cat submandibular gland during perfusion with sulphate Locke s solution *Experientia* 1968b *24* 919-920 (19 28)

Petersen, O.H. & J H Poulsen, Transmembrane secretory potentials and electrolyte transients in salivary glands. In *Exocrine Glands.* Eds. Botelho, S Y., F.P Brooks & W B Shelley Philadelphia University of Pennsylvania press, 1969 pp 3-20 (14 19 33)

Petersen O.H., J H Poulsen & N A. Thorn Secretory potentials, secretory rate and water permeability of the duct system in the cat submandibular gland during perfusion with calcium-free Locke s solution *Acta physiol. scand.* 1967 *71* 203-210. (19 29 31 32, 38 40)

Rasmussen, H Cell communication, calcium ion and cyclic adenosine monophosphate. *Science* 1970 *170* 404-412. (35)

Proverbio F J W L. Robinson & G Whittembury Sensitivities of (Na K^+)–ATPase and Na extrusion mechanisms to ouabain and ethacrynic acid in the cortex of the guineapig kidney *Biochim. Biophys. Acta* 1970 *211* 327 336. (30)

Schachter M., Control of blood flow in the salivary glands. In *Secretory mechanism of salivary glands*. Eds Schneyer L.H & C.A Schneyer New York – London. Academic Press. 1967 pp 209 219 (39)

Schneyer L.H. & Yoshida, Y Secretory potentials in rat submaxillary gland *Proc. Soc Exp. Biol Med* 1969 *130* 192 196 (12)

Schneyer L.H. & C.A. Schneyer Electrolyte and water transport by salivary gland slices. *Am. J Physiol* 1962, *203* 567 571 (13)

Schneyer L.H. & C.A. Schneyer Salivary secretion in the rat after ouabain *Am. J Physiol.* 1965a, *209* 111 118. (Table I 12)

Schneyer L.H & C.A. Schneyer Membrane potentials of salivary gland cells of rat. *Am. J Physiol* 1965b *209* 1315-1310 (Table II 13)

Schneyer L.H & C.A Schneyer In *Secretory mechanism of salivary glands*. New York – London Academic Press. 1967 pp 32 55 & p 99 (17 25)

Selinger Z. E Naim & M Lasser ATP-dependent Ca^{2} -uptake by microsomal preparation from rat parotid and submaxillary glands. *Biochim. Biophys. Acta* 1970 *203* 326-334 (32 34 37)

Sten-Knudsen, O Is muscle contraction initiated by internal current flow? *J Physiol (Lond.)* 1960 *151* 363-384 (11)

Strömblad B.C.R. Gaseous metabolism of the normal and denervated submaxillary gland of the cat. *J Physiol (Lond)* 1959 *145* 551 561 (38)

Thaysen J H., In H H Ussing, P Kruhoffer J H Thaysen & N.A. Thorn *The alkali metal ions in biology* Handbuch der experimentellen Pharmakologie. Ergänzungswerk Bd 13 Berlin-Göttingen-Heidelberg Springer 1960. pp 424-438 (9)

Thaysen J H. & N.A. Thorn Excretion of urea, sodium potassium and chloride in human tears. *Am J Physiol* 1954 *178* 160-164 (24)

Thaysen J H N A. Thorn & I L. Schwartz Excretion of sodium potassium, chloride and carbon dioxide in human parotid saliva. *Am. J Physiol.* 1954 *178* 155-159 (9 24)

Ullrich K.J Permeabilität der Kortikalen Nephronabschnitte in Beziehung zu Transportvorgängen und Struktur In *Sekretion und Exkretion* Ed K.E Wohlfahrt Bottermann Berlin-Heidelberg-New York Springer Verlag. 1965 pp 392-403 (28 29)

Ussing, H.H The alkali metal ions in isolated systems and tissues. In *The alkali metal ions in biology* Handbuch der experimentellen Pharmakologie Ergänzungswerk. Bd 13 Berlin Springer 1960 pp 112 129 (29)

Whittembury G., Relationship between sodium extrusion and electrical potentials in kidney cells. In *Electrophysiology of Epithelial Cells* ed Giebisch, G. & Frömter E Stuttgart New York Schattauer Verlag, 1971 pp 153-186 (30 36)

Whittembury G & J Fishman, Relationship between cell Na extrusion and transtubular absorption in the perfused toad kidney The effect of K⁺ ouabain and ethacrynic acid *Pflugers Arch.* 1969 *307* 138-153 (30)

Whittembury G & F Proverbio Two modes of Na extrusion in cells from guinea pig kidney cortex slices. *Pflügers Arch* 1970 *316* 1 25 (30)

Yoshimura, H. Secretory mechanism of saliva and nervous control of its ionic composition. In *Secretory mechanism of salivary Glands.* Eds. Schneyer L.H & C.A. Schneyer New York London Academic Press 1967 pp. 56-74 (17)

Yoshimura, H. & Y Imai Studies on the secretory potential of acinal cell of dog s submaxillary gland and the ionic dependency of it. *Jap. J Physiol.* 1967 *17* 280-293 (13 17)

Yoshimura, H., T Inoue Y Imai & F Yoshimura Studies on mechanism of salivary secretion. *Jap. J Physiol.* 1962, *12* 1 1 (28)

Young, J.A. & C.J Martin, The effects of sympatho- and parasympathomimetic drugs on the electrolyte concentration of primary and final saliva of the rat submaxillary gland. *Pflugers Arch.* 1971 *327* 285-302 submitted for publication. (9 26)

Young, J.A., E Frömter E. Schögel & K F Hamann A microperfusion investigation of sodium resorption and potassium secretion by the main excretory duct of the rat submaxillary gland. *Pflügers Arch.* 1967 *295* 157 172. (29)

Young, J.A. & E. Schögel Micropuncture investigation of sodium and potassium excretion in rat submaxillary saliva. *Pflügers Arch* 1966 *291* 85-98. (9 28)

Öbrink, K.J & M Waller Deviation from steady state concentrations of potassium in gastric juice. *Acta physiol scand* 1966 *suppl* 277 234 (24)

Index

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 382

Linkoping University Medical Dissertations
No 7

CYCLIC AMP AND CALCIUM IONS IN MECHANICAL AND METABOLIC RESPONSES OF SMOOTH MUSCLES, INFLUENCE OF SOME HORMONES AND DRUGS

BY
ROLF G G ANDERSSON



Linköping 1972

ISBN 91 7222 010 4

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 382

Linkoping University Medical Dissertations
No 7

CYCLIC AMP AND CALCIUM IONS IN MECHANICAL AND METABOLIC RESPONSES OF SMOOTH MUSCLES, INFLUENCE OF SOME HORMONES AND DRUGS

BY
ROLF G G ANDERSSON

Department of Pharmacology School of Medicine
Linköping Sweden



Linköping 1972

This survey is based on the following papers which will be referred to as I—VIII

I Andersson R and E. Mohme Lundholm Metabolic actions in intestinal smooth muscle associated with relaxation mediated by adrenergic α and β-receptors Acta physiol scand. 1970 79 244—261

II Andersson R. Role of cyclic AMP and Ca^{++} in metabolic and relaxing effects of catecholamines in intestinal smooth muscle. Acta physiol scand. 1972. 85 312—322

III. Andersson, R. Role of cyclic AMP and Ca^{++} in mechanical and metabolic events in isometrically contracting vascular smooth muscle. Acta physiol scand 1972

IV Andersson R. and K Nilsson Cyclic AMP and calcium in relaxation in intestinal smooth muscle Nature New Biology 1972 238 119—120

V Andersson, R Cyclic AMP as a mediator of the relaxing action of papaverine, nitroglycerine, diazoxide and hydralazine in intestinal and vascular smooth muscle. Acta Pharmacol 1972

VI Andersson, R E. Mohme Lundholm N Svedmyr and N Vamos Relaxing and metabolic actions of ACTH in rabbit colon Acta physiol scand 1971 81 11—17

VII Andersson, K.E R Andersson and P Hedner Cholecystokinetic effect and concentration of cyclic AMP in gallbladder muscle in vitro Acta physiol scand. 1972 85 511—516

VIII Andersson, R. Relationship between cyclic AMP phosphodiesterase activity calcium and contraction in intestinal smooth muscle Acta physiol scand 1972

Contents

Introduction

The original aim of the present investigation was to study the relation ship between cyclic AMP and relaxation of smooth muscle mediated by stimulation of adrenergic β-receptors. As there is increasing evidence that the contraction relaxation cycle of smooth muscles as well as other kind of muscles, is regulated by the concentration of free myoplasmic Ca^{++} the connection between cyclic AMP and Ca^{++} in intestinal and vascular smooth muscle was studied (II and III) These studies indica ted that cyclic AMP stimulated a Ca^{++} binding process in smooth musc le. The isolation of a Ca^{++} binding microsomal fraction from intestinal smooth muscle where the Ca^{++} binding was stimulated by catecholami nes and cyclic AMP is reported in paper IV and in this survey The possibility that other agents than catecholamines, such as papaverine, nitroglycerine, hydralazine and diazoxide relaxed smooth muscle by cyclic AMP was investigated in paper V

Since an increased content of cyclic AMP relaxed smooth muscle the contrary relationship i.e. a reduction of the level was expected to lead to a contraction of smooth muscle. The contracting action of some hor mones and drugs such as the C-terminal octapeptide of cholecystokinin (C8—CCK) and imidazole was found to be combined with a reduced cyclic AMP content (VII and VIII) Other contracting agents (carbacholine K^{+} adrenaline histamine) had however a biphasic action on the cyclic AMP content an initial decrease was followed by a secondary increase. The latter effect was associated with an inhibition of the phosphodiesterase activity The secondary rise of the concentration of cyclic AMP and the inhibition of phosphodiesterase activity were dependent on the presence of Ca^{++} (VIII) Carbacholine released Ca^{++} from the mic rosomal fraction and simultaneously reduced its adenyl cyclase activity (this survey) Some evidence for a compartmentilization of the cyclic AMP regulating enzymes to this microsomal fraction of the smooth muscle cell was found (V) The possibility is discussed that the local con centration of cyclic AMP in this fraction is a regulating factor for Ca^{++} binding or release, and thereby has an effect on the contraction relaxation cycle of smooth muscle

Previous investigations

The main topic for this investigation has been the relationship between cyclic AMP and the contraction relaxation cycle in smooth muscle. Cyclic AMP also mediated some metabolic effects of catecholamines and other drugs such as stimulation of carbohydrate metabolism and activation of phosphorylase a. These effects were observed before cyclic AMP was discovered and are of interest to relate since they initiated some of the studies on cyclic AMP in smooth muscle reported in this survey

Relationship between relaxation, cyclic AMP and other metabolic processes in smooth muscle.

An association between the relaxation induced by catecholamines and an increased formation of lactate was early demonstrated by Mohme Lundholm (1953) in different kinds of smooth muscle. A part of this action was attributed to an increased glycogenolysis (Lundholm and Mohme Lundholm 1957) An activation of phosphorylase a (Axelsson, Bueding and Bülbring 1961 Mohme Lundholm 1962) was also reported to be associated with a relaxation induced by adrenaline.

Rall and Sutherland (1958) showed that the increased formation of phosphorylase a in liver and skeletal muscle induced by adrenaline was mediated by cyclic AMP They suggested that cyclic AMP was involved in smooth muscle relaxation too (Sutherland and Rall 1960)

Mohme Lundholm (1963) demonstrated that cyclic AMP activated (tracheal) smooth muscle phosphorylase a in vitro Butcher et al (1965) reported that adrenaline after 10 min. of incubation had increased the cyclic AMP content 6 times in rat uterus Bueding et al (1966) found that the relaxing action of adrenaline in taenia coli of guinea pig was associated with a moderate (about 30 %) increase in cyclic AMP The type of adrenergic receptor that was stimulated was not characterized, however Dobbs and Robison (1968) and Robison et al (1971) later demonstrated that β-receptor stimulation in myometrial tissue was associated with an increased cyclic AMP content observations that were confirmed by Polacek and Daniel (1971) and Triner et al. (1971)

In taenia coli of guinea pig Bueding et al. (1966) found however no simultaneous increase in hexosephosphate content despite an increase of the cyclic AMP content In an earlier study Bueding et al (1962) had reported a dissociation between relaxation and phosphorylase a activation in taenia coli They later demonstrated (Bueding et al 1967) that the relaxing effect of adrenaline on taenia coli was not associated with an ac

tivation of the carbohydrate metabolism but was combined with an increase in the ATP and CrP content This is an effect earlier found by Lundholm and Mohme Lundholm (1962) to be produced by adrenaline in vascular smooth muscle under some experimental conditions.

A possible explanation for the divergent results regarding catecholamine induced relaxation and metabolic effects in intestinal smooth muscle was the observation by Ahlquist and Levy (1959) that the relaxation was mediated both by adrenergic α and β receptors. This observation was later confirmed by Furchgott (1960) and Bucknell and Whitney (1964) Jenkinson and Morton (1965 1967) subsequently demonstrated that stimulation of α receptors in intestinal smooth muscle was at 20 C, associated with an increase in permeability to potassium ions an effect not present on β-receptor stimulation They suggested that the biophysical and biochemical background of the two kinds of relaxation was divergent, a suggestion sustained by the studies of Brody and Diamond (1967) Setekleiv (1967) working with taenia coli at 37 C, was not able to confirm the results of Jenkinson and Morton regarding an increased K^+ permeability after adrenaline, using a normal Krebs solution. In solutions with high potassium ([K] 29.5 and 59 mM) however he observed an increased potassium efflux after adrenaline (Setekleiv 1970) Andersson and Mohme Lundholm (1968 1969) found, by systematic variation of the experimental conditions that in taenia coli from guinea pig and in rabbit colon adrenergic α and β-receptor mediated relaxation differed in a number of respects.

Relationship between contraction cyclic AMP and other metabolic reactions in smooth muscle.

The relationship found between relaxation, cyclic AMP changes and carbohydrate metabolism of smooth muscle produced by catecholamines evoked the question if there was any corresponding relationship during contraction of smooth muscle.

On studying glycogenolysis in bovine mesenteric artery and guinea pig uterus contracted isotonically by adrenaline Lundholm and Mohme Lundholm (1957) got results indicating that relaxation was associated with an increased glycogenolysis whereas contraction retarded glycogenolysis In experiments on bovine tracheal smooth muscle and rabbit ileum contraction with carbacholine increased glycogenolysis in the first preparation, but had no effect in the latter muscle electrical stimulation increased the glycogen breakdown, however (Lundholm and Mohme Lundholm 1963 b) Contraction of smooth muscle was therefore not regularly combined with increased glycogenolysis which instead sometimes was reduced

Isotonic contraction of tracheal smooth muscle by carbacholine was combined with an activation of phosphorylase a (Mohme Lundholm 1962) In rat uterus contraction by different drugs (serotonin acetyl choline, bradykinin oxytocin and barium chloride) was associated with an activation of phosphorylase a too (Brody and Diamond 1967) When bovine mesenteric arteries were contracted isometrically by adrenaline or Ca^{++} there was evidence that the lactate production was stimulated both by increased energy consumption associated with the contractile process and by a direct mechanism (Lundholm and Mohme Lundholm 1963 Mohme-Lundholm and Vamos 1967 Andersson, e t a l 1971) In mesenteric arteries contracted isotonically by K^+ the lactate production was reduced initially in isometrically contracted arteries lac tate production was markedly increased at the time of contraction

That a reduction of the cyclic AMP content in smooth muscle might lead to a contractile action was first discussed by Robison, Butcher and Sutherland (1967) who speculated that an adrenergic α receptor stimu lation might reduce the cyclic AMP content and thereby stimulate the contractile process. They pointed out however that some of the changes in the carbohydrate metabolism produced by adrenaline in smooth muscle where not commensurate with a decrease in the cyclic AMP con tent. The possibility that the cyclic AMP system was compartmentilized within the smooth muscle cell was discussed. The idea that contraction of smooth muscle might be related to a reduced cyclic AMP content got some support when it was reported that adrenergic α receptor stimula tion was combined with a reduction of the cyclic AMP content in some tissues (Turtle and Kipnis 1967 Robison and Sutherland 1970 Anders son and Mohme-Lundholm 1970) This effect was also reported to oc cur in vascular tissue (Volicer and Hynie 1971) and in homogenate of uterine smooth muscle (Triner e t a l 1971) in which tissues adrenergic α receptors mediate a contracting action Angiotensin reduced cyclic AMP formation in vascular tissue too (Volicer and Hynie 1971) On the other hand Polacek and Daniel (1971) reported that isotonic contrac tion of the β-receptor blocked rat uterus by adrenaline was not combi ned with cyclic AMP changes. Neither was the contracting effect of oxy tocin combined with any change of cyclic AMP in rat uterus (Robison, e t a l. 1971)

Bartelstone e t a l. (1967) found that theophylline in low concentra tions could increase the contracting effect of noradrenaline on rat aorta They therefore suggested that α receptor stimulation was related to an increase in the cyclic AMP level Robison e t a l (1971) have raised some objections to this suggestion however

The related findings between contraction cyclic AMP changes and carbohydrate metabolism indicate a rather complex relationship

Enzymes regulating the cyclic AMP content of smooth muscle.

According to the original concept of Sutherland and Rall (1960) cyclic AMP *is synthetized by adenyl cyclase and hydrolyzed by phosphodieste* rase. The cyclic AMP content was thought to depend on a balance bet ween these two processes Newer studies have confirmed this suggestion (Robison e t a l 1971) although the enzyme systems involved are more complex than originally thought and other ways of elimination can exist. Cyclic AMP will under normal conditions diffuse out of the cell to a limited degree This type of elimination can, when the membrane permeability is increased reduce the tissue content of cyclic AMP (Kuo and Renzo 1969) Murad Rall and Vaughan (1969) found indications that cyclic AMP may be metabolized to other compounds than 5 AMP

A d e n y l c y c l a s e. Sutherland and Rall (1960) and Sutherland Rall and Menon (1962) demonstrated adenyl cyclase activity in aorta intestinal and uterine smooth muscle of the dog and guinea pig The pre sence of adenyl cyclase in uterine smooth muscle was confirmed by But cher e t a l (1965) Dobbs and Robison (1968) Szego and Davis (1969) and Triner Overweg and Nahas (1970) Robison e t a l (1971) in intes tinal smooth musle by Bueding e t a l (1966) Andersson and Mohme Lundholm 1970) Robison et al (1971) and in arterial smooth muscle by Schonhofer e t a l (1970) Triner e t a l (1971) Volicer and Hynie (1971) paper III The potency of catecholamines to stimulate myomet rial adenyl cyclase was in the order isoprenaline>adrenaline>norad renaline corresponding to their ability to relax the muscle (Dobbs and Robison 1968)

P h o s p h o d i e s t e r a s e The presence of an enzyme hydrolyzing cyclic AMP was demonstrated in smooth muscle tissue (uterus and in testine) by Sutherland and Rall (1958 1960) Since theophylline was an effective inhibitor of the enzyme (Sutherland and Rall 1960 Butcher and Sutherland 1962) and was able to potentiate the relaxing action of isoprenaline in intestinal smooth muscle besides having a relaxing ac tion of its own (Lundholm Mohme Lundholm and Svedmyr 1966) the possibility that smooth muscle tone might be influenced by changes of the phosphodiesterase activity was considered. Kukovetz and P ch (1970) demonstrated phosphodiesterase in coronary vessels and made the important observation that many vasodilating drugs inhibited the phos phodiesterase activity of coronary vessels. Triner e t a l (19 1) further observed that the ratio between the phosphodiesterase and adenyl cycla se activity decreased with decreasing diameter of the artery n the dog

Recent findings indicate that the phosphodiesterase enzyme is of a complex nature and can be influenced in different ways by ions and drugs No complete picture of this enzyme can be given at present but the observations indicate the importance of phosphodiesterase as a target of drugs for regulation of the cyclic AMP level in several tissues.

Phosphodiesterase has been isolated in different molecular forms some of which were also capable of hydrolyzing cyclic guanosine 3 5 monophosphate (Thompson and Appleman 1971) or uridine 3 5 mononophosphate (Klotz and Stock 1971) From rabbit tissue, Monn and Christiansen (1971) electrophoretically isolated seven different forms of phosphodiesterase of which as many as four were simultaneously present in the same organ. Uzunov and Weiss (1972) isolated with an electrophoretic chromatographic technique five different forms from rat brain Phosphodiesterases exist which have different K_m and V_{max} when tested for their ability to hydrolyze cyclic AMP (Beavo Hardman and Sutherland 1970 Thompson and Appleman 1971) Amer (1971) reported that the phosphodiesterase activities with different K_m and V_{max} values were interconvertible (PDE I and PDE II) PDE II was stimulated by adrenaline, an effect blocked by phentolamine. Phosphodiesterase from brain was associated with a protein activator On removal of this factor the enzyme became relatively inactive (Cheung 1970)

Cheung (1967) reported that phosphodiesterase from brain was inhibited by Cu^{++} and some other divalent cations (Zn^{++} Ca^{++}) in a rather high concentration (2 mM) Kakiuchi, Yamazaki and Teshima (1971) found, however that Ca^{++} did activate brain phosphodiesterase a discrepancy which was partly solved when it was reported that only one of 5 forms of phosphodiesterase from brain was activated by Ca^{++} (Uzunov and Weiss 1972)

Calcium and smooth muscle function.

Vertebrate smooth muscle reacts by contraction or relaxation to changes of their environment A very large number of drugs and allergens, changes of the milieu exterieur variations of other physical conditions such as temperature, light and stretch all will influence the myogenic activity of smooth muscle. Many papers covering this field of research have been published. Reviews of different aspects of this topic have recently been published (Somlyo and Somlyo 1968 1970 Bülbring et al 1970 Ruegg 1971) At present all available facts cannot be summarized in some unifying hypothesis Increasing evidence indicates, however that variations in the free myoplasmic concentration of calcium is of fundamental importance for the myogenic activity of

smooth muscle (Edman and Schild 1962, Daniel 1963 Hurwitz e t a l 1967 Rüegg 1971) as well as that of skeletal and cardiac muscle (Weber e t a l 1963 Ebashi and Endo 1968 Nayler 1967) According to Ebashi and Ebashi (1964) and Ebashi and Endo (1968) Ca^{++} ions act by binding to the troponin tropomyosin system and upon saturation of this system with Ca^{++} it fails to exert the inhibiting effect on the interaction of myosin and actin as it does in absence of Ca^{++} This system has been demonstrated in smooth muscle as well (Sparrow e t a l 1970 Ruegg 1971) It must be pointed out however that the evidence for the role of Ca^{++} in the contraction relaxation cycle of smooth muscle is still indirect *In vitro* the presence of Ca^{++} is necessary for the contraction of smooth muscle (Edman and Schild 1962) In different glycerinated smooth muscle fiber preparations the threshold concentration of Ca^{++} for a contractile effect is 1.8×10^{-7}M and maximum tension is reached at 1×10^{-6}M (Filo Bohr and Ruegg 1965 Schadler 1967 Ruegg 1971) The total calcium content of smooth muscles is much higher about $2-5 \times 10^{-3}$M (Bauer e t a l 1965 II) Only a very small part of the total Ca^{++} is directly involved in the contractile process

The concentration of myoplasmic Ca^{++} in smooth muscle is probably regulated by more than one mechanism It has been suggested that in taenia coli from guinea pig the spike charge during the action potential is carried by Ca^{++} (Bulbring and Kuriyama 1963) but this mechanism is probably not present in vascular smooth muscle (Graham and Keatinge 1972) Besides some smooth muscle contracts or relaxes even in the absence of action potentials whether the cell membrane is polarized (Somlyo and Somlyo 1968) or depolarized (Evans, Schild and Thesleff 1958) The existence of different Ca^{++} pools in smooth muscle has therefore been postulated (Hinke 1965 Hurwitz e t a l 1967 van Bremen e t a l 1972) which releases or binds Ca^{++} The cell elements corresponding to the different binding sites for Ca^{++} are not defined The plasma membrane (Bianchi 1969) sarcoplasmic reticulum — which however is sparse in most kind of smooth muscle (Nagasawa and Suzuki 1967) — and the mitochondria all bind or accumulate Ca^{++} (Ebashi and Endo 1968 Haugaard *e t a l* 1969) *and are probable candidates. From uterine* and intestinal smooth muscle a microsomal protein fraction has been isolated which bind Ca^{++} (Carsten 1969 Batra and Daniel 1971 Andersson e t a l. 1971 b Hurwitz e t a l 1972)

If cyclic AMP influences smooth muscle function it is probable that there is a connection between Ca^{++} and cyclic AMP metabolism Hess e t a l. (1966) found that adrenergic β-receptor blocking agents reduced the Ca^{++} uptake of cardiac sarcoplasmic reticulum Entman e t a l (1969) in following up these observations found that isoprenaline and glucagon

stimulated adenyl cyclase activity of cardiac sarcoplasmic reticulum and increased the Ca^{++} binding an effect also produced by cyclic AMP it self

In the following my own published and some unpublished results, and observations of other investigators are discussed with regard to probable relationship between the contraction relaxation cycle of smooth muscle, cyclic AMP and Ca^{++}

Methods

The principal procedures used in the present study were (1) registration of muscle tension (2) determination of cyclic AMP content (3) estimation of phosphodiesterase, phosphorylase and adenyl cyclase activities (4) spectrophotometric quantitations of muscle levels of ATP CrP and hexosephosphates (5) determination of the tissue content of calcium using an atomic absorption technique (6) uptake studies of labelled Ca of isolated microsomes.

The following isolated organs were used

R a b b i t c o l o n m u s c l e covered by the united taenia was removed, cut up longitudinally and washed in Krebs buffer at 20 C. The mucosal layer was carefully separated from the layer of muscle. From the part covered with taenia similarly shaped specimens 8—10 mm wide and 12—15 mm long and weighing 75—100 mg were prepared.

B o v i n e m e s e n t e r i c a r t e r i e s (Ø 5—7 mm) were dissected free approximately 20 min after slaughter and transferred to a Dewar flask containing Krebs buffer (37 C) which was continuously gassed with a mixture of 95 % O_2 and 5 % CO_2. The arteries were cleaned of adventitious tissue as far as possible without damaging the tunica media. They were cut up longitudinally and divided into 8—10 mm broad pieces weighing 80—120 mg

G a l l b l a d d e r s f r o m g u i n e a p i g were dissected out after killing the guinea pigs by a blow to the neck The gallbladders were divided in three parts, and the rings thus obtained were cut open to form strips of gallbladder tissue. The mucosa was removed carefully under a stereo microscope. The specimens obtained were 2—3 mm wide and 8—12 mm long weighing 10—25 mg

Suspension solutions.

The Krebs solution used had the following composition (mM) 122 NaCl 4 7 KCl 2 6 $CaCl_2$ 1.2 $MgCl_2$ 15 4 $NaHCO_3$ 1.2 KH_2PO_4 and 11.5 glucose.

In the experiments in which the preparations were depolarized with K ions, the composition of the suspension solution was (mM) 126.7 KCl 2 6 $CaCl_2$ 1 2 $MgCl_2$ 15 4 $NaHCO_3$ 1 2 KH_2PO_4 and 11.5 glucose. When Ca free solution was used $CaCl_2$ was excluded from the normal Krebs solution The suspension solutions were continuously aerated with 95 % O_2 + 5 % CO_2 at 37 C.

Mechanical recording technique.

Isometric measurement Muscle preparations were mounted in special holders (Lundholm and Mohme Lundholm 1966) In these holders the distance between the points of attachment of the preparations could be varied. The muscular tension was recorded by a Grass force-displacement transducer FT 03 C on a Grass polygraph model 5

Isotonic measurement The shortening during the isotonic contraction was registered by means of Clevite Brush isotonic muscle transducer Model 33—03—981 The output signal was recorded on a Grass polygraph model 5

Procedure for the estimation of cyclic adenosine 3 5' monophosphate.

Cyclic AMP was determined by a specific and sensitive enzymatic m t hod described by Kakiuchi and Rall (1968) The estimation of cyclic AMP was based on its ability to stimulate the conversion of dephospho phosphorylase to phosphorylase catalyzed by extracts from dog liver Dephosphophosphorylase and the dog liver supernatant fraction were prepared essentially as described previously (Kakiuchi and Rall 1968)

Tissue extraction The tissues processed were frozen rapidly in freon 11 containing solid CO_2. Muscular tissues were then homogenized in a chilled glass homogenizer (—20 C) after addition of 2 ml of a solution of 0 1875 N HCl in 60 % ethanol After the first grind 13 ml redistilled H_2O was added and the tissue was rehomogenized The homogenates were then centrifuged chromatographed and lyophilized according to the original method After these procedures the apparent cyclic AMP was, in mean 81 6±1.5 % of the original

Assay of cyclic AMP The estimation of the cyclic AMP concentration was then performed essentially as in the original method of Kakiuchi and *Rall* The incubation medium was, *however changed to* the following composition 167 mM Tris (pH 7 4) 30.3 mM $MgSO_4$ 17 4 mM ATP 8 9 mM caffein and 1 4 mM sodium EDTA (pH 7 4) The composition of the enzyme systems was determined empirically for each batch of enzymes, so the difference between standard curves from different batches was as small at possible. The recovery of cyclic AMP added to a muscle homogenate was determined for this enzyme system For the addition of 3 p mol cyclic AMP the recovery was 95.0±1 8 % for 6 p mol 100.3±1 7 % and for 9 pmol 96 5±1 6 %.

Determination of phosphodiesterase, phosphorylase and adenyl cyclase activity

Phosphodiesterase The activity of the enzyme phosphodiesterase was determined with radioactively labelled cyclic 3 5 AMP as substrate according to the method of Poch (1971) ^{14}C-cyclic 3 5 AMP was used as substrate in the enzyme reaction, and unlabelled 5' AMP was added to the reaction mixture to prevent the conversion of labelled 5 AMP to adenosine. The product of the phosphodiesterase reaction, 5 AMP was precipitated from the substrate, cyclic 3 5 AMP by the $ZnSO_4$ Ba (OH_2) method (Krishna et al 1968) The efficiency of the precipitation was controlled by cromatography after eluation of the different spots the radioactivity was determined by liquid scintillation technique

The accuracy of the method was the following Differences between cpm in duplicate estimation of phosphodiesterase activity were found to be less than ± 1.5 % of the calculated rate of hydrolysis and less than ± 2.5 % of the calculated percentage of change of phosphodiesterase activity by drugs

Phosphorylase activity was estimated according to the method of Bueding et al (1962) and is described in paper I The amount of G 1 P formed in the phosphorylase reaction was determined by an enzymatic method using the enzymes phosphoglucomutase and glucose 6 phosphate dehydrogenase. The recovery of glucose-1 phosphate by this method was 98.5±1 9 % in the concentration interval 3—60 nmole G 1 P

The adenyl cyclase activity of the microsomal fractions of colon muscle was determined according to Osnes et al (1972)

Adenosinetriphosphate, creatinephosphate and hexosephosphate determination.

For determination of ATP CrP and hexosephosphate levels muscles were frozen in freon 11 and solid CO_2 at —80 C and homogenized at 0 C in 8 vol 6 % perchloric acid and after neutralization with K_2CO_3 the mentioned phosphate compounds were determined enzymatically G 1 P and G-6-P according to Bergmeyer and Klotzch (1962) F-6-P according to Hohorts (1962) ATP and CrP according to Lamprecht and Stein (1962)

Determination of tissue level of calcium.

The muscle was digested in ultrapure HNO_3 and both samples and standards were adjusted to 0.5 N HNO_3 and strontium was added up to 1 % to reduce the interference due to the phosphate present in the muscle as described by Parker (1963) The concentration of Ca in the samples was determined on a Unicam PYE SP 90 A atomic absorption spectrophotometer at 4226 A

^{45}Ca accumulation studies in microsomal fractions.

Rabbit colon muscle were fractioned as described by Carsten (1969) The pellet obtained after the centrifugation at 40 000 x g for 90 min was homogenized in a solution containing 0 08 M NaCl and 0 005 M Na oxalate, pH 7 0 The suspended pellet was placed on a sucrose density gradient consisting of 35 45 and 55 % sucrose. Centrifugation at 45 000 x g for 2 h in a MSE Super speed 65 revealed three protein layers In paper IV the layer at a sucrose gradient of 35 % was used The protein concentration of the layer was determined by the method of Weichselbaum (1946) using bovine serum albumin as a standard The uptake of ^{45}Ca by the microsomal fraction was performed in the incubation mixture containing $1\,25 \times 10^{-4}$ M Ca and described in paper IV The rate of calcium uptake was measured by filtering aliquots at specified time intervals through Millipore filters (0 45 μ) Aliquots of the filtrates were counted in a Packard Tri Carb liquid Scintillation counter using Instagel^{R} (Packard) as scintillation fluid

Statistical methods.

In the statistical analysis the t test was used in all investigations Of the statistical symbols used $t = \frac{\text{mean}}{\text{standard error of the mean}}$ P = the probability of an observed effect of difference having been due to chance. The different degrees of statistical validity were indicated as follows P = 0 01—0 05 statistically probable P = 0 001—0 01 statistically very probable P<0 001 statistically significant.

In some paper the linear regression and the correlation coefficient were calculated on a Hewlett Packard 9100B calculator using the standard formulas (Freund 1962)

Results

Influence of catecholamines on the cyclic AMP content of intestinal and vascular smooth muscle in relation to their relaxing effect.

Following up the original observation by Ahlquist and Levy (1959) that relaxation of intestinal smooth muscle was mediated both by α and β receptors Jenkinson and Morton (1965 1967) Brody and Diamond (1967) Andersson and Mohme Lundholm (1968 1969) and Bowman and Hall (1970) found indications that the mechanism of relaxation was different depending on which receptor type was stimulated. A schematic representation of the observed differences is collected in table II paper II

In experiments on taenia coli and colon muscle of the rabbit, L-phenylephrine in a concentration of 1×10^{-6}g/ml almost specifically stimulated α receptors as the relaxation was completely blocked by the α antagonist dibenamine Higher concentrations of phenylephrine ($>2\times10^{-6}$g/ml) relaxed the muscle by stimulating β-receptors as well. Relaxation by specific stimulation of β-receptors was produced by L isoprenaline in a concentration of 5×10^{-7}g/ml as the relaxation was totally blocked by the β-blocking agent sotalol ($1\ 2\times10^{-5}$g/ml) Isoprenaline in a higher concentration activated α receptors as well Adrenaline or noradrenaline relaxed the muscle by stimulation of both α and β-receptors. In rabbit colon muscle the β-receptors was most sensitive toward these catecholamines in guinea pig taenia coli the α receptors were most sensitive (Andersson and Mohme Lundholm 1969)

It was evident from these experiments that unless the receptors stimulated by catecholamines were characterized it would be very difficult to correlate mechanical and metabolic events in intestinal smooth muscle. When adrenaline or noradrenaline was used, the receptor type stimuated might depend on the concentration of the agonist and the species studied The sensitivity of the β-receptors in rabbit colon moreover showed seasonal variations so that the threshold concentration for a relaxing action was lower during the summer than the winter months (Andersson and Mohme Lundholm 1969) It is probable that most of the earlier controversy regarding the relation between relaxation and metabolic effects in intestinal smooth muscle (Lundholm, Mohme Lundholm and Svedmyr 1966 Bueding e t a l 1967) is due to the fact that the observed metabolic effects have not been obtained by stimulation of one single receptor type.

Metabolic effect associated with α receptor mediated relaxation in *rabbit colon.*

In table II paper II the characteristics of relaxation and metabolic ef fects elicited by stimulation of α or β-receptors in rabbit colon are col lected. Relaxation following α-receptor stimulation was rapid and al most complete. There were no metabolic effects present at the onset of relaxation (5 sec) but after some delay (1 min) there was a moderate reduction of the cyclic AMP content a reduction of the phosphorylase a activity and a reduction of the hexose phosphate and lactate contents, but an increase in the ATP and CrP concentration. The reduction of the carbohydrate metabolism indicated that the decrease of the cyclic AMP level had metabolic consequences The time relationship between re laxation and cyclic AMP changes in the smooth muscle did not indicate a causal relationship between the two events The probable mecha nism of the cyclic AMP-decreasing action in these tests is discussed on page 37

The α receptor mediated relaxation seemed to be dependent on the existence of K^+ or Na^+ gradient over the cell membrane (Andersson and Mohme Lundholm 1969) Bowman and Hall (1970) reported that membrane stabilizers (quinidine) blocked the α-effect. They also found that exogenous ATP produced a relaxation resembling that of α stimu lation, an action which they thought sustained the suggestion by Bue ding et al (1967) that the relaxing action of adrenaline depended on an increased content of high energy phosphate compounds in the musc le In our experiments, however the relaxation occured before the ATP content rose. It is therefore more probable that the increase in ATP was caused by or parallel to the relaxation. The mechanism of the α receptor mediated relaxation in smooth muscle is also discussed later (page 37)

Beta receptor mediated relaxation in smooth muscle.

When studying relaxation and contraction in rabbit colon and mesente ric arteries different degrees of activity have been compared but not the complete resting and contracted state of the preparation. Both kind of muscles have a basal intrinsic myogenic activity and only upon total in *hibition of the energy production do mesenteric arteries relax comple* tely (Lundholm and Mohme Lundholm 1966) In most tests the initial tension of the preparation was increased by a contracting drug to ob tain a pronounced relaxing effect. As discussed on page 29 contracting drugs will after some delay increase the cyclic AMP level a relaxing drug increased this level still more. In the reported experiments rela tive changes of the contractile state have therefore been related to cor responding changes of the cyclic AMP level but there have been no

studies of the cyclic AMP changes upon complete relaxation or maximal contraction of the muscles When relaxation is expressed in per cent, this is in relation to the muscle tension produced by carbacholine (rabbit colon) or by histamine (mesenteric artery) but an eventual influence on the intrinsic myogenic activity is not included

R a b b i t c o l o n. On specific stimulation of adrenergic β-receptors *in rabbit colon there was a significant increase in the cyclic AMP* content within 10 sec before the relaxation had been manifest. Metabolic actions attributable to an increased cyclic AMP level such as phosphorylase a activation, an increase in the hexosephosphates (G 1 P G-6 P and F 1 6-DP) and elevated lactate contents were also present. When the relaxation had been more prominent the increase in cyclic AMP and other metabolites was more marked With increasing concentration of isoprenaline there was a correlation between the degree of relaxation and the increase in the cyclic AMP content (II) Beta receptor stimulation also reduced the ATP and CrP contents. After 10 min, however the ATP reduction was no longer present despite the fact that the cyclic AMP content was still increased (II) The metabolic actions, as the relaxing effect of isoprenaline, were blocked by the adrenergic β-receptor blocking agent sotalol (Andersson and Mohme Lundholm 1968 I II)

Rabbit colon was also relaxed by ACTH (VI) which increased the cyclic AMP content and stimulated carbohydrate metabolism ACTH *did not, however reduce the ATP-content of the muscle. Since the re*laxation and metabolic effects were competitively blocked by the adrenergic β-receptor blocking agent sotalol it seems probable that β-receptors were involved. In human isolated bronchi ACTH had a relaxing action too and increased the cyclic AMP content and phosphorylase a activity Relaxation was also blocked in this tissue by sotalol (Andersson Bergh and Svedmyr 1972) ACTH stimulation of β-receptors in smooth muscle contrasted to findings in adipose tissue (Butcher e t a l 1968 Robison et al 1971) where the lipolytic effect of ACTH was non competitively blocked by adrenergic β-blockers.

V a s c u l a r s m o o t h m u s c l e. In the isolated bovine mesenteric artery no or a very small relaxing action of isoprenalin or adrenaline was present until α receptors were blocked by dibenamine. In the α-blocked preparation an increase of cyclic AMP preceded relaxation and metabolic effects (phosphorylase a activation, increase in hexose phosphates ATP reduction) similar to those of intestinal muscle appeared Relaxation and metabolic effects were blocked by β-receptor antagonists (III)

In most experiments the vessels were contracted by histamine to visualize the relaxation. Contracting drugs had however a cyclic AMP increasing effect themselves (page 28) It was found that in vessels not contracted by histamine, isoprenaline increased the cyclic AMP level with $7.9 \pm 1.8 \times 10^{-10}$ mol/g from a control value of $5.3 \pm 1.7 \times 10^{-10}$ mol/g whereas in the contracted preparations the corresponding values were $4.0 \pm 0.5 \times 10^{-10}$ mol/g and $19.0 \pm 3.0 \times 10^{-10}$ mol/g Calculated as precentage the cyclic AMP increasing action of isoprenaline was 149.1 ± 34.0 % of the control muscle whereas in contracted muscles the increase was 21.1 ± 2.6 % (difference 128 ± 34 % $p < 0.01$ $n = 7$)

An increase of cyclic AMP in vascular tissue when β-receptors are stimulated is in agreement with findings of Triner et al (1971) in their experiments on arteries from the dog and those of Volicer and Hynie (1971) and Ericsson (1972) on rat aorta. In homogenated vascular tissue (Sutherland and Rall 1960 Triner et al 1971 and Schonhofer et al 1971) catecholamines did not however stimulate cyclic AMP formation whereas it did in a homogenate from myometrial tissue (Triner et al 1971) These authors have suggested that the β-receptor is very loosely associated with adenyl cyclase in vascular smooth muscle.

Alpha receptor stimulation in intact vascular smooth muscle was combined with a decreasing component of cyclic AMP formation. In intact bovine mesenteric artery the α and β-receptor stimulating actions of adrenaline balanced each other so well that no change of the cyclic AMP content was present until one of the receptors were blocked (III) The possibility that the same balance exist between stimulating and inhibiting impulses in the cyclic AMP metabolism of both homogenates and intact tissue ought to be considered

Role of Ca^{++} in the metabolic effects of isoprenaline and cyclic AMP

It was pointed out in the introduction that if cyclic AMP influences smooth muscle function there ought to be a connection between Ca^{++} — and cyclic AMP metabolism Following up this hypothesis, the action of Ca^{++} isoprenaline and cyclic AMP was studied in Ca^{++} poor smooth muscle preparations (Andersson and Mohme Lundholm 1969 b II and III)

The total Ca^{++} content of the rabbit colon muscle was reduced to about 20 % (from 5.9 to 1.3×10^{-3} mol/kg wet weight) by incubation in a Ca^{++} free Krebs buffer containing 2×10^{-4} M EDTA for 60 min. The contractile action of carbacholine was lost after about 30 min (II) The Ca^{++} reduction in bovine mesenteric arteries was even more pronounced by treatment with Ca^{++} free Krebs buffer and EGTA The total

Ca-content was reduced to 10 % of the initial value (from 12.1 to 1.2×10^{-3} mol/kg) (III) The muscle preparations were thus not Ca^{++} free but Ca^{++} poor

In the Ca^{++} poor colon and mesenteric artery there was a reduction of cyclic AMP content phosphorylase a activity and content of hexose phosphates the ATP content was reduced too (II III) Addition of 3×10^{-3}M Ca^{++} to the Ca^{++} poor colon preparation caused muscle contraction (Andersson et al 1972 d) and cyclic AMP content and phosphorylase a activity increased toward basal values Ba^{++} in the same concentration reproduced the myogenic and metabolic effects of Ca^{++} poor preparation (II)

Isoprenaline and ACTH still increased the cyclic AMP content and activated phosphorylase a in the Ca^{++} poor intestinal muscle and isoprenaline had the same effect in the Ca^{++} poor vascular preparation The ATP reducing action of isoprenaline in normal muscles, however was reversed to an increasing effect in the Ca^{++} poor muscles. The reduction of ATP induced by cyclic AMP itself as well as that of some other relaxing drugs such as papaverine, nitroglycerine, hydralazine and diazoxide, was also inhibited in the absence of Ca^{++} (V) In rat uterus Polacek and Daniel (1971) have also reported that Ca^{++} deprivation did not reduce the cyclic AMP increasing effect of adrenaline.

The reduction of cyclic AMP content and the phosphorylase a activity that followed α receptor mediated relaxation in rabbit colon was not present in the Ca^{++} poor preparation (II) On the other hand the initial reduction of the cyclic AMP content following α receptor mediated contraction in vascular smooth muscle was still present and was prolonged in the Ca^{++} poor preparation (III)

On page 41 the relationship between the cyclic AMP and Ca^{++} metabolism is further discussed.

Influence of phosphodiesterase inhibitors and activators on the relaxing action of catecholamines in smooth muscle.

Theophylline is a rather effective inhibitor of phosphodiesterase (Butcher and Sutherland 1969) and potentiates many actions of drugs that are supposed to be mediated by cyclic AMP (Rall and West 1963 Robison et al 1971) Lundholm et al. (1966) reported that under some circumstances the relaxing effect of catecholamines was potentiated by theophylline. Andersson and Mohme Lundholm (1968 1969) later demonstrated that relaxation mediated by β-stimulation was potentiated, whereas that mediated by α stimulation was not. The potentiating action of theophylline or caffeine on the relaxing action of catecholamines

in some smooth muscle preparations has been found by others too (Ariens 1967 Dobbs and Robison 1968 Levy and Wilkenfeld 1968 Kawasaki et al 1969 Robison et al. 1971 Triner et al 1971)

On the other hand Bowman and Hall (1970) showed that high concentrations of theophylline (0 4—1 mM) in some cases reduced or counteracted the relaxing effect of adrenaline or cyclic AMP on rabbit intestine It is recognized that the methylxanthines may have other actions than a phosphodiesterase inhibiting action (Sutherland, Robison and Butcher 1968) such as release of Ca^{++} from the sarcoplasmic reticulum (Weber 1968 Blinks et al 1972) which might explain some of these observations

Puromycine which according to Appleman and Kemp (1966) inhibits phosphodiesterase, potentiated the relaxing action of adrenergic β-receptor stimulation but not that of α receptor activation in taenia coli of rabbit (Andersson and Mohme Lundholm 1969) Diazoxide, another phosphodiesterase inhibitor potentiated the β-receptor response in rabbit ileum (Wilkenfeld and Levy 1969)

Imidazole stimulates phosphodiesterase according to Butcher and Sutherland (1962) It reduced the cyclic AMP content of rabbit colon and contracted the muscle (VIII) Bueding and Bulbring (1967) found that the relaxing action of adrenaline on taenia coli of guinea pig was inhibited by imidazole. Andersson and Mohme Lundholm (1969) reported that the relaxation mediated both by α and β-receptor stimulation was inhibited by imidazole in taenia coli of rabbit. In tests on rabbit ileum Wilkenfeld and Levy (1969) got evidence however that the β receptor mediated relaxation was reduced by imidazole, whereas the α receptor mediated response was not influenced. Bowman and Hall (1970) found however that both α and β-receptor mediated relaxation of rabbit small intestine was reduced by imidazole.

Cyclic AMP formation in relation to the relaxing action of other drugs.

Drugs which inhibits the phosphodiesterase activity The role of cyclic AMP in smooth muscle relaxation may be further elucidated by investigating the effect of other relaxing drugs than the catecholamines Brody and Diamond (1967) failed to find activation of the phosphorylase a activity when rat uterus was relaxed by nitroglycerine and thus suggested that phosphorylase activation was not related to uterine relaxation Levy and Wilkenfeld (1968) reported however that the action of nitroglycerine was potentiated by theophylline. An important step in correlating the action of coronary vessel dilating drugs with cyclic AMP metabolism was made when Kukovets and co-

workers (Kukovetz Poch and Juan 1969 Kukovetz and Poch 1970) demonstrated that the relaxing action of papaverine, with regard to time and dose, was related to an inhibition of phosphodiesterase in tests on bovine coronary vessels. Many other drugs with a relaxing action such as theophylline dipyridamol iproveratril prenylamine carbochromen and oxyfedrine also inhibited the phosphodiesterase activity A good correlation between the relaxing action of papavarine and its ability to inhibit the phosphodiesterase activity was also found in rat arteries by Triner et al (1971)

Papaverine was found to increase the cyclic AMP content of rabbit colon in a concentration that had relaxing effect (Andersson et al 1971) In contrast to these reports are the findings of Polacek, Bolan and Daniel (1971) who reported that papaverine theophylline and diazoxide in low concentrations relaxed rat uterus with minimal or no elevation of the cyclic AMP level Only in higher concentrations did theophylline increase the cyclic AMP level

The relationship between the relaxing action of papaverine, nitroglycerine diazoxide and hydralazine on the cyclic AMP content, phosphodiesterase activity and role of Ca^{++} were studied in rabbit colon and bovine mesenteric arteries (V) All these drugs increased the cyclic AMP content in a concentration which had a relaxing effect, decreased the ATP content and stimulated to some degree the phosphorylase a activity Papaverine, nitroglycerine and diazoxide inhibited the phosphodiesterase activity of smooth muscle in pharmacologically active concentrations but hydralazine had only an inhibiting effect in a much higher concentration.

The ATP reducing effect of papaverine and nitroglycerine was Ca^{++} dependent but the cyclic AMP increasing action was not. The increase in the cyclic AMP content, reduction of ATP and relaxing effect showed similar degree of correlation after papaverine and isoprenaline. The change in the phosphorylase a activity and relaxation were positively correlated after isoprenaline but negatively correlated after papaverine (V) A possible explanation for these observations was that the cyclic AMP formation was influenced differently by papaverine in the parts of the cell where phosphorylase a activation and the Ca^{++} dependent ATP ase stimulation took place. To test this hypothesis the action of papaverine on the phosphodiesterase activity of different cell fractions was tested. Papaverine activated the soluble (cytoplasmic) form of the enzyme but markedly (to 70—90 %) inhibited the form of the enzyme bound to the microsomal fraction which bound Ca^{++} When nitroglycerine was tested on the phosphodiesterase activity of the same

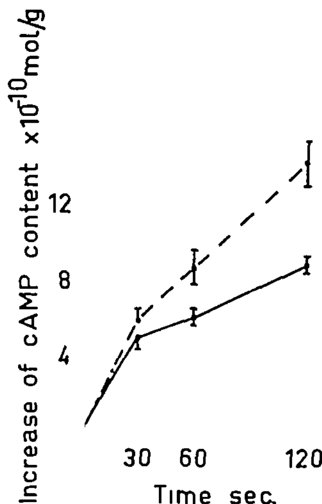


Fig 1

Effect f PGE₁ on cy li AMP content in rabbit colon muscle (5×10^{-7}g/ml) —— nd h ma bronchial muscl (2×10 g/ml) ‐‐‐‐‐‐ ——, contracted by carbacholine (1.7×10 7g/ml) The relax ng action tarted fter about 15 s and reached a m xim m of about 100 afte 60 s. Mean ± S E. (n=6 $p < 0.01$)

cell fractions essentially similar results were obtained (V) These experiments indicated that 1 probably different forms of phosphodiesterase exist in smooth muscle 2 cyclic AMP might be compartmentilized in the cell this last suggestion is further discussed on page 44

Other cyclic AMP increasing drugs The relaxing *action of C8 CCK on the sphincter Oddi from the cat was also combined* with an increase of the cyclic AMP content. It was of special interest that C8 CCK stimulated the phosphodiesterase activity despite its cyclic AMP increasing action (Andersson e t a l. 1972 a)

The prostaglandins are able both to relax and contract smooth muscle (Euler 1967 Bergstrom and Samuelsson 1967) PGE_1 relaxed rabbit colon muscle at a concentration of 5×10^{-7}g/ml and increased the concentration of cyclic AMP The time course of this effect is shown in fig 2. The increase in cyclic AMP preceded the relaxation. In human isolated bronchi PGE1 had corresponding actions even in a concentration as low as 2×10^{-8}g/ml (Fig 1)

Åberg and Andersson (1972) demonstrated that the local anaesthetic agent mepivacaine increased the cyclic AMP content both of taenia coli from guinea pig and rat portal vein the L-isomer being more potent than the D form in the latter preparation. The cyclic AMP increasing action of the L-isomer was blocked by sotalol Mepivacaine had both a relaxing and contracting component in these kind of smooth muscles. The part of the relaxing effect of L-isomer that was inhibited by sotalol was attributed to the increase in cyclic AMP

Myogenic and metabolic effects of exogenously added cyclic AMP and its derivatives.

The effects of exogenously added cyclic AMP are of great interest for determining the role of cyclic AMP in mechanical and metabolic effects of drugs on smooth muscle. However to get an effect from exogenously added cyclic AMP the nucleotide must be added in about 1000 times higher concentration than normally found in the tissue. This is probably due to its slow penetration of the cell membrane (Robison et al 1965) and rapid hydrolysis by phosphodiesterase There is the difficulty however that the high concentration of exogenously added cyclic AMP needed to produce myogenic and mechanical effects in smooth muscle might produce "unspecific" effects related to 5 AMP or adenosine accumulation when cyclic AMP is hydrolyzed by the tissue. The dibuturyl derivative is more stable and may therefore offer an advantage (Robison et al 1971) Cyclic AMP like the other nucleotides, has a vasodilating action *in vivo* as reported by Hashimoto et al. (1964) That the vasodilation was combined with more specific metabolic effects of cyclic AMP was found by Levine and Vogel (1965) Levine Cafferata, McNally (1967) later reported that i.v infused cyclic AMP reduced the intestinal motility and tone in man and dog *in vivo* Cyclic AMP and its dibuturyl derivative which is more slowly hydrolyzed by smooth muscle phosphodiesterase (Moore, Iorio and McManus 1968) had a relaxing action on different kind of isolated smooth muscle. This has been confirmed by many authors. Cyclic AMP also reduces the contracting action of different drugs These actions have been found in tests on isolated bronchi (Moore Iorio and McManus 1968 Svedmyr

et al. 1970) intestinal smooth muscle (Andersson and Mohme Lundholm 1968 1970 Levine 1968 Kim Shulman and Levine 1968 Bowman and Hall 1970) urinary bladder (Eggena Walter Schwartz 1968) myometrium (Dobbs and Robison 1968 Mitznegg Heim and Meythaler 1970 Triner Overweg and Nahas 1970 Robison et al 1971 Polacek Bolan and Daniel 1971) arterial smooth muscle (Gebert et al 1969 Andersson 1972 Triner et al 1971) and vas deferens of the guinea pig (Lamble 1970)

The specificity of the relaxing action of cyclic AMP in relation to other nucleotides has been questioned and attributed to the adenosine moiety or 5' AMP (Kim et al 1968 Robison et al 1971) which are smooth muscle relaxing agents (Drury 1936) The evaluation of the relaxing action of adenosine is however complicated by the fact that this nucleotide can increase the cyclic AMP content in some tissues i.e. brain slices (Rall and Sattin 1970)

Using rabbit colon we found cyclic AMP to be a more potent and long lasting relaxing agent than 5 AMP or some other tested cyclic nucleotides. It was also much more potent than dibuturyl cyclic AMP confirming the observations of Levine (1968) In human bronchi (Andersson Bergh and Svedmyr 1972) and rat uterus (Triner et al 1971) the dibuturyl derivative was however more potent than cyclic AMP The relaxing action of cyclic AMP in rabbit colon was dose dependent, and associated with the same metabolic effects as produced by β-receptor stimulation such as phosphorylase a activation, increase of carbohydrate metabolism and reduction of ATP In contrast, 5 AMP only decreased the ATP-content The coefficient of correlation between the degree of relaxation and phosphorylase a activation was of the same magnitude after isoprenaline and cyclic AMP (I) but this correlation was not present after 5 AMP The corresponding coefficient of correlation between relaxation and ATP reduction was however of similar magnitude for isoprenaline cyclic AMP and 5' AMP

When determining the cyclic AMP content after adenosine (0.5mM) or 5 AMP (5mM) induced relaxation, it was found to be increased with 12 4±4 % respectively 24 0±5.3 % (n=8 $p<0.02$) compared with the control muscle. These results indicate that some part of the relaxing effect of exogenously added 5 AMP and adenosine in rabbit colon can be ascribed to cyclic AMP

Cyclic AMP and calcium binding of microsomal protein fractions.

Beta receptor stimulation, papaverine nitroglycerine diazoxide and hydralazine which relaxed smooth muscle and increased the cyclic AMP

content all produced a Ca^{++} dependent reduction of the ATP content similar to that produced by cyclic AMP After isoprenaline where the time-course of the latter effects was studied the reduction was only present during the first minutes but not after 10 min although the cyclic AMP content was still increased and the muscle relaxed at this time. In the absence of Ca^{++} isoprenaline increased the ATP content probably as a consequence of the stimulation of the carbohydrate metabolism (II) The relaxing drugs consequently stimulated a time limited Ca^{++} dependent ATP-consuming process In skeletal and cardiac muscle the sarcoplasmic reticulum which binds Ca^{++} during utilization of ATP fulfils these criteria (Hasselbach 1964 Ebashi and Endo 1968 Weber et al 1966) Entman et al (1969) and Shinebourne et al (1970) have reported that isoprenaline and cyclic AMP increased the Ca^{++} binding of the sarcoplasmic reticulum from dog heart Sulakhe and Dhalla (1970) were unable to confirm this results. The observations of Entman and cowokers were confirmed however by our laboratory (Nilsson and Andersson, unpublished) If cyclic AMP could stimulate a Ca^{++}-binding process in smooth muscle this effect might explain the relaxing effect of drugs that elevated the cyclic AMP level

The structure corresponding to sarcoplasmic reticulum is sparse in most kinds of smooth muscle (Lane and Rhodin 1964 Nagasawa and Suzuki 1967) The existence of a longitudinal smooth sarcotubular system has, however been described in intestinal smooth muscle (Lane 1965 1967) In vascular smooth muscle, Somlyo Devine and Somlyo (1972) found a sarcoplasmic reticulum as well A Ca^{++} binding microsomal fraction has been isolated from intestinal smooth muscle (Miyazaki et al 1965 Andersson et al 1971 Hurwitz et al 1972) and also from myometrial tissue (Carsten 1969 Batra and Daniel 1971 a, b) Carsten (1969) isolated 3 different fractions after ultracentrifugation on a sucrose density gradient. Electron microscopy of these fractions established the vesicular nature of the material The material from myometrial tissue isolated by Batra and Daniel (1971 a) was not vesicular however

The isolation technique of Carsten (1969) used on a homogenate from rabbit colon muscle gave three Ca^{++} binding microsomal fractions which contained no dectable succinate dehydrogenase activity but showed adenyl cyclase and phosphodiesterase activity When incubated in a suitable incubation medium the fractions rapidly bound Ca^{++} The Ca^{++} binding was increased by isoprenaline, which moderately stimulated the cyclic AMP formation (Fig 9) and by cyclic AMP itself Andersson et al 1971 IV) The Ca^{++} binding of the fractions and the effect of cyclic AMP was maximal already after

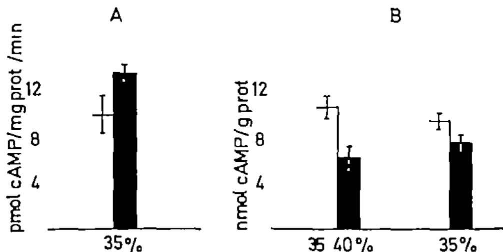


Fig 2 a.

Adenyl cyclase activity f microsomal fraction from rabbit col muscle without (open bars) and w th isoprenaline (5×10− g/ml filled bars) Th activity was determined f 10 min. according to Omes et al (1972) Mean ± S E (n=8) p<0.05 f r differences in p ired tests on the same m sle h mogenat

Fig 2 b

F rmation of cyclic AMP in microsomal fraction i absence (open bars) and presence of carbacholine (2×10⁻⁸ g/ml; filled bars) Incubation was carried out at 37 C f r 3 min a mixture containing 25 mM Tris buffer pH 7 4 0 35 mM MgCl₂, 0 05 mM CaCl₂, 0.35 mM ATP a d 10 mM theophylline The microsomal fracti ns sed, were those isolated at a sucrose concentration of 35 / r 35—4 /. Cycli AMP wa ana lysed acco ding t Kakiuchi and Rall (1968) Mean ± S E (=8 p<0 05, f diff ences in paired tests on the same muscle homogenate)

60 sec. The action of isoprenaline was completely blocked by sotalol (1 2 ×10⁻⁴g/ml) Papaverine (4×10⁻⁴g/ml) which markedly inhibited the phosphodiesterase activity also increased the Ca⁺⁺ binding This effect was most pronounced in the fraction at 35—45 % sucrose (V) ACTH which relaxed smooth muscle by a stimulation of β-receptors, also in creased the Ca⁺⁺ binding The Ca⁺⁺ binding was associated with an increased rate of ATP hydrolyses (Nilsson and Andersson to be published)

After the preliminary results of these experiments had been publi shed (Andersson e t a l. 1971) Batra and Daniel (1971 b) reported that cyclic AMP (1×10⁻³—10⁻⁸M) did not increase the Ca⁺⁺ binding of myometrial microsomes Adrenaline increased the Ca⁺⁺ uptake first after 5—10 min. The authors did not support the hypothesis that cyclic AMP was involved in the relaxing action of the catecholamines or other drugs in smooth muscle. The experimental conditions especially the

composition of the incubation solution of Batra and Daniel (1971) differed in many respects from that used in paper IV Thus the sucrose ATP Ca^{++} and Mg^{++} concentrations in their experiments were much higher than used in the present investigation. Furthermore, their solution lacked K^{+} and Na^{+} ions. The total Ca^{++} uptake was also much higher in the myometrial microsomes than in those of intestinal smooth muscle. For these reasons the Ca^{++} uptake of microsomes from rabbit colon was restudied using the incubation solution of Batra and Daniel (1971 a) The Ca^{++} uptake per mg protein was larger than that reported in paper IV Cyclic AMP in a concentration of 1×10^{-6}M reduced Ca^{++} uptake during the first minute. After 10 min however the uptake was greater primarily because the Ca^{++} uptake curve showed a tendency to decrease with time in experiments without cyclic AMP (Nilsson and Andersson unpublished)

It is obvious that under the experimental conditions of Batra and Daniel cyclic AMP did not have the same effect on the Ca^{++} uptake as under our experimental conditions One may only speculate about the reason for this difference. Since the basal Ca^{++} uptake was much higher under the experimental conditions of Batra and Daniel it is not improbable that the maximum binding capacity was reached and could not be further increased by cyclic AMP The high Ca^{++} concentration used by Batra and Daniel (1971) and the high ATP concentration — about 14 times the content of colon muscle — might produce this maximal binding of Ca^{++} Further investigation of the action of cyclic AMP on the Ca^{++} binding in fractions of smooth muscle under different experimental conditions are needed to answer this question Another question raised by Batra and Daniel (1971 a) needs to be studied too whether the binding capacity is enough to reduce the Ca^{++}-concentration below 1.8×10^{-7}M in the incubation solution i.e. below the threshold concentration for contraction in glycerinated muscle (Filo Bohr and Ruegg 1965)

The mechanism by which cyclic AMP increased the Ca^{++} binding in the microsomal fraction can only be a matter of speculation. In the sarcoplasmic reticulum of skeletal muscle, Ca^{++} is both bound to the membrane and pumped into the vesicles, especially in the presence of oxalate (Ebashi and Endo 1968) Cyclic AMP activates a protein kinase which phosphorylates proteins (Walsh et al. 1970) Ca^{++} is probably partly bound to the phosphate groups in the membranes (Feinstein 1969) Cyclic AMP by increasing bound phosphate groups might stimulate the binding of Ca^{++} to the membranes

The eventual role of the Ca^{++} binding process in the contraction relaxation process for the action of cyclic AMP is discussed later

Smooth muscle contraction and cyclic AMP

It was suggested in the introduction (page 3) that the relationship bet ween contraction of smooth muscle and the changes of the carbohydrate metabolism (lactate production, glycogenolysis phosphorylase a activa tion) was rather complex and dependent on 1 the kind of smooth muscle studied, 2 how contraction was initiated 3 whether isotonic or isomet ric contraction was studied When planning the studies on the rela tionship between contraction and cyclic AMP changes these findings were taken in consideration

In the following section the more unequivocal results about the rela tionship between smooth muscle contraction and cyclic AMP changes are first presented before the more complicated relationship are discus sed.

Phosphodiesterase activators

Amer (1969) reported that cholecystokinin increased the phospho diesterase activity of a tissue homogenate. Paper VII reported that upon isometric contraction of the isolated guinea pig gallbladder by the C-terminal octapeptide of cholecystokinin (C8—CCK) the increase of ten sion was preceded by an activation of phosphodiesterase and a reduc tion of cyclic AMP content. Agents, such as catecholamines glucagon and theophylline and dibuturyl cyclic AMP which probably increase the cyclic AMP content counteracted the contracting action of C8—CCK It was further found that C8—CCK relaxed the sphincter Oddi of the cat and increased the total cyclic AMP content despite an increase in phosphodiesterase activity (Andersson *e t a l* 1972 b) After C8—CCK treatment contraction and relaxation of smooth muscle was corre lated with a corresponding decrease or increase in cyclic AMP content respectivelv

Table I

Mechanic l nd metabol ff t f prostaglandin E_2 (1 3×10 $^{-7}$g/ml) on gui ea pig gallbladder Mea ± S E. Number f tests 8—10.

Time afte PGE$_2$ addition	AMP nm l/g wet weight ontr l	test	Change f PDE compared with control (%)	Incre se of tension Dyn
30 sec		—0 40 ± 0 0	+66.3 ± 16.0	5.5 ± 1 1
	1 ± 0 1	$p < 0.001$	$p < 0.01$	$p < 0.001$
180 sec		—0 42 ± 0 1	+54 1 ± 18.9	23.3 ± 4.9
	1.2 ± 0 1	$p < 0.01$	$p < 0.05$	$p < 0.001$

C8—CCK, being a peptide might diffuse through the cell membrane very slowly and a direct influence on the intracellular metabolic processes is therefore questionable. Catecholamines and sympathetic stimulation are reported to realase prostaglandins (Gilmore Vane and Wyllie 1968) which markedly influence the cyclic AMP content in different tissues (Robison et al. 1971) It was therefore of interest to study the action of prostaglandins on gallbladder smooth muscle and sphincter Oddi As show in table I PGE_2 contracted the gallbladder activated phosphodiesterase and reduced the cyclic AMP content. In sphincter Oddi PGE_2 relaxed the muscle increased the cyclic AMP content and activated phosphodiesterase i.e. mimiced the effects of C8—CCK (Andersson et al 19 2 b) Whether the actions of C8—CCK are mediated by one of the prostaglandins remains however to be established.

Imidazole, another phosphodiesterase activating agent contracted rabbit colon while increasing the phosphodiesterase activity and reduced the cyclic AMP content (VIII) Imidazole, furthermore potentiated the contracting effect of carbacholine and Ca^{++} ions in rabbit colon both regarding rate and amplitude of tension development (VIII)

Isotonic and isometric contraction by K^+ in vascular muscle. Lundholm and Mohme Lundholm (1965) detected marked differences in the influence of K^+ on lactate production during the initial phase of isotonic and isometric contraction of bovine mesenteric arteries There was a decrease in the former case and an increase in the latter

Tabl II

Met bolic changes i bovin mesenteric rtery 5 ml fter isotonic r isometric contraction with Krebs solution contal ing 126.7 mM K^+ Mean ± S E. (n=7—8) den tes $p<0.05$

	Isotonic		Isometric	
	cont l	test	control	test
phosph. a activity /a	25.3 ± 5.8	—1.3 ± 3.8	16 9 ± 1 7	+10.5 ± 2.9*
G — 6 — P nmol/g	23 ± 3	+1 ± 3	21 ± 4	+17 ± 5
ATP nmol/g	490 ± 128	—68 ± 24	619 ± 131	—192 ± 67
CrP nm l/g	304 ± 53	—83 ± 38	361 ± 48	—218 ± 67*

This initiated a comparison between the cyclic AMP changes and other metabolic effects during isotonic and isometric contraction of mesenteric arteries by K^+ The preparations were pretreated by sotalol This was done to inhibit any stimulation of adrenergic β-receptors by catecholamines released from sympathetic nerve endings The results are presented in table II and fig 3

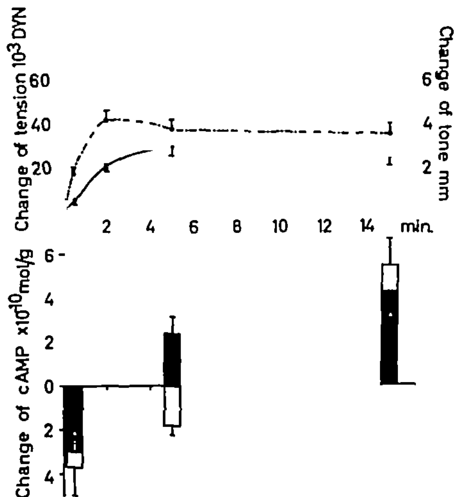


Fig 3
Effect f K+—ions (136.7 mM) on muscle tone and cyclic AMP content f mesenteric arteries. Changes during isometric and isotonic contraction i indicated by filled bars (———) and open bars (o——o) respectively The basal values f cyclic AMP were at isometric conditions $8.3 \pm 1.0 \times 10^{-10}$ mol/g and t isotonic conditions $7.2 \pm 0.9 \times 10$ mol/g Mean ± S E. (n=3 p<0.07)

During isotonic contraction by K^+ there was a significant decrease of the cyclic AMP content between 0.5—5 min When the shortening of the muscle had been maximal there was a rise in the level There were no significant changes of the phosphorylase a activity or of G 6-P content the ATP and CrP contents were moderately reduced. During isometric contraction the basal cyclic AMP values were of the sam magnitude Only after 0 5 min there was a reduction of the cyclic AMP level th nucleotide level was raised after 5 and 15 min however After 5 min both the phosphorylase a activity and content of hexosephosphates were raised. The reduction in the high energy phosphate compounds was mo re marked during isometric than isotonic contraction These experiments indicated that the contractile effect of K^+ was associated both with a reducing and an increasing component of the cyclic AMP level and that, during isotonic contraction the increasing component appeared lat-r

Isometric contractions by carbacholine phenylephrine, histamine and K^+ Studies of the time-course of the cyclic AMP changes during isometric contraction of rabbit colon by carbacholine and K^+ (VIII) or of the bovine mesenteric artery by phenylephrine adrenaline or histamine on the β-receptor blocked preparation, (III) demonstrated the same two components Before the tension development had started (10—15 sec) there was a reduction of th cyclic AMP content in both kinds of smooth muscle. When the tension had reached a maximum 120 sec after addition of the drugs the cyclic AMP content was increased as were the phosphorylase a activity and the hexosephosphates.

The ability of the catecholamines to influence the content of cyclic AMP and other metabolic effects in the β-receptor blocked mesenteric art ry was inhibited by the adrenergic α blocking drug dibenamine and that of histamine by the antihistamine drug promethazine (III) In rabbit colon the effect of carbacholine on the cyclic AMP content and phosphorylase a activity was totally inhibited by atropine. Sotalol only reduced these effects of carbacholine (VIII The cyclic AMP reducing effects of K^+ observed after 30 sec was blocked by atropine (8.3×10^{-7}g/ml) In absence of atropine the decrease was $1\,2 \pm 0.3 \times 10^{-10}$mol/g ($p<0\,05$) and in the presence there was an increase of $1\,1 \pm 0.3 \times 10^{-10}$mol/g ($n=5$ $p<0.05$) from a basal value of $4\,9 \pm 0.5 \times 10^{-10}$mol/g

The results indicated that not only the action of adrenergic α receptors but also of some other contracting agents consisted of both a reducing and an increasing action on the cyclic AMP level The increasing action occured rather late during the contractile process especially during isotonic contraction. The contractile process is probably re

gulated by the myoplasmic Ca^{++} concentrations Since the cyclic AMP content of rabbit colon was influenced by variations in the extracellular Ca^{++} concentration (II) the influence of carbacholine K^{+} histamine and adrenergic α receptor stimulation (phenylephrine and adrenaline) was studied in the Ca^{++} poor rabbit colon (VIII) and bovine mesenteric artery (III)

In Ca^{++} poor preparations the contractile action of the tested agents were naturally absent After 60—120 sec of incubation with the contracting agents the cyclic AMP level was reduced in the Ca^{++} deficient preparations whereas at the same time in normal muscles there was an increase of the nucleotide (VIII) The reduction of the ATP content was blocked (III)

The Ca^{++} dependence of the cyclic AMP increasing action of these contractile agents was contrary to the corresponding effect of adrenergic β-receptor stimulation which was not Ca^{++} dependent (II) This indicated that the mode of action of the two categories of drugs was different.

The two modes of action on the cyclic AMP content were also noted when studying the local anaesthetic agent mepivacaine. This agent had both a relaxing and contracting component depending on the concentration of drug and initial muscle tension The cyclic AMP increasing effect was partly dependent on a stimulation of adrenergic β-receptors (blocked by sotalol) and partly dependent on the contractile effect (absent in the Ca^{++}-deficient muscle) (Åberg and Andersson 1972)

The time difference in the cyclic AMP changes during isotonic and isometric contraction observed after contraction by K^{+} might explain some of the divergent results reported in the initial survey considering the prior findings of the relationships between contraction and carbohydrate metabolism in smooth muscle.

It is obvious that the mode of registration of the contractile process is of importance for th observed metabolic processes a fact that some investigators are not aware of as the kind of registration (isotonic and isometric) is not always stated The reason for the difference is not clear Ruegg (1971) has discussed the probable existence of a tonic mechanism in invertebrate smooth muscle, which was minimally energy consuming and present after prolonged soaking in calcium free solution Whether a corresponding tonic mechanism exist in vertebrate smooth muscle is not documented but mammalian smooth muscle can attain different degrees of isotonic shortening without changes of the energy consumption (Lundholm and Mohme Lundholm 1962)

The findings that the secondary rise of the cyclic AMP level after K+ was faster during isometric than isotonic contractions could be regarded as a consequence of the fact that the increase in myoplasmic Ca^{++} might be more rapid during isometric than isotonic contraction The greater decrease of the higher energy phosphate compounds in the former contraction also support this suggestion. According to van Bremen et al (1979) K+ increases the Ca^{++} influx through the cell membrane During isotonic contraction the area of the cell membrane will probably decrease as the muscle shortens whereas it is constant during isometric contraction. These geometrical changes caused by K+ treatment might influence the magnitude of the Ca^{++} influx and the myoplasmic Ca^{++} concentration.

Phosphodiesterase inhibition and smooth muscle contraction.

The mechanism by which contracting agents and adrenergic β receptors agonists influenced the cyclic AMP level was different in some respects The initial effect of contracting agents was a reduction. An increase became evident first, after that the tension or shortening was maximal The relaxing action of isoprenaline, however was preceded by an increase of cyclic AMP In the Ca^{++} poor muscle, contracting agents did not increase the cyclic AMP level whereas the action of β-receptor stimulation was unchanged in the absence of Ca^{++} Specific receptor antagonists blocked the increasing action on the cyclic AMP level of β-receptor agonists On the other hand blocking agents such as atropine (in rabbit colon) or dibenamine (in vascular smooth muscle) blocked the cyclic AMP decreasing action of contracting agents even in the Ca^{++} poor preparations. It has been reported that in a homogenat of heart, acetylcholine reduced the adenyl cyclase activity (Murad, Chi Rall and Sutherland 1962) In a homogenate of myometrial tissue, α receptor stimulation also reduced the cyclic AMP formation (Triner et al 1971) These considerations indicated that it was most probable that the reducing action on the cyclic AMP level induced by contracting agents was due to an inhibition of adenyl cyclase, provided that an activation of phosphodiesterase was not present. The effect on the phosphodiesterase activity of contracting agents seemed therefore worth to investigate.

All the investigated contracting agents (carbacholine, K+ phenylephrine, adrenaline, histamine) had a moderate but probably significant reducing effect on the phosphodiesterase activity both in rabbit colon and bovine mesenteric arteries (III VIII) The inhibition of the phosphodiesterase activity preceded the increase in cyclic AMP content. In fact, 10 sec after addition of carbacholine to rabbit colon there was an inhi

bition of the phosphodiesterase activity despite a decrease of the cyclic AMP content, which probably indicated a reduction of both the adenyl cyclase and the phosphodiesterase activities (VIII) There was a dose dependent increase of tension and cyclic AMP content, and an inhibition of phosphodiesterase activity in rabbit colon 60—120 sec after the addition of carbacholine (VIII) Contraction of bovine mesenteric artery by phenylephrine reduced the phosphodiesterase activity too an effect blocked by dibenamine (III) Mepivacaine decreased th phosphodiesterase activity in guinea pig taenia coli in a concentration that contracted the muscle and increased its cyclic AMP content (Åberg and Andersson 1972) The inhibition of the phosphodiesterase activity by carbacholine (VIII) and by mepivacaine (Åberg and Andersson 1972) and by phenylephrine in bovine mesenteric artery (III) was not present in the Ca^{++} poor muscle.

Phosphodiesterase activity of smooth muscle homogenate.

These findings initiated a study of the Ca^{++} sensitivity of the smooth muscle phosphodiesterase. In planning these experiments it was thought that it might not be the total tissue content of Ca^{++} that influenced the phosphodiesterase activity but rather the free fraction, just as in the case of the contractile process With EGTA and Ca^{++} in the proportions calculated according to Ebashi and Endo (1968) tissue homogenate of rabbit colon was incubated with different concentrations of "free" Ca^{++} Even in a concentration as low as 5×10^{-7}M calcium ions inhibited the phosphodiesterase activity The inhibition was more pronounced at a cyclic AMP concentration of 1×10^{-7}M than at a concentration of 1×10^{-4}M (VIII) In additional experiments the Ca^{++} sensitivity was tested on the phosphodiesterase activity from different cell fractions prepared as described in paper IV None of these fractions showed, however any sensitivity to Ca^{++} It is therefore probable that some other factor in combination with Ca^{++} may be of importance for the phosphodiesterase inhibition. Such factors are known. For example Cheung (1971) and Kakiuchi and Yamazaki (1970) have isolated a phosphodiesterase activating factor from brain.

The Ca dependent inhibition of the phosphodiesterase activity amounted at most to 18 % of the control activity in the diluted homogenate Papaverine and nitroglycerine reduced the enzyme activity to 70—100 % in the Ca^{++}-binding microsomal fraction (V) in concentrations that raised the cyclic AMP content to about the same level as the studied contracting agents. Although the effect of the contracting agent on the cyclic AMP content was correlated with respect to time, dose and Ca^{++} dependence to the inhibition of the phosphodiesterase activity the

question can be raised whether the increase in nucleotide content was dependent on the enzyme inhibition alone or whether some other mechanism such as an activation of adenyl cyclase was involved too

This latter assumption is supported by the following facts Isoprenaline increased the cyclic AMP level more in uncontracted mesenteric arteries than in those contracted by histamine (page 16) If histamine had raised the cyclic AMP level only through inhibition of the phosphodiesterase activity a contrary relationship could be expected.

Ba^{++} added to the Ca^{++} poor rabbit colon had a contracting action and raised the cyclic AMP level as much as addition of the equimolar amount of Ca^{++} (paper II) In a homogenate of colon muscle Ba^{++} had however no significant inhibitory action on the phosphodiesterase activity (unpublished experiments)

General discussion

Factors influencing the cyclic AMP content in smooth muscle.

The results reported in this survey indicate that the cyclic AMP content of intact smooth muscle can be influenced by several different mechanisms involving both an effect on the adenyl cyclase and the phosphodiesterase activities.

The exact means by which the cyclic AMP content was influenced in the different cases have not been clarified in detail however A change of the cyclic AMP level at a constant total phosphodiesterase activity has been interpreted as an effect on the adenyl cyclase activity and vice versa This assumption is probably too simple since some drugs — i e. C8—CCK in sphincter Oddi — may increase both enzyme activities at the same time The possibility that the cyclic AMP content is changed in different directions in separate compartments of the cell must be considered too An unchanged total tissue level of cyclic AMP is no proof that the distribution of cyclic AMP within the cell compartments was unchanged. Thus, both papaverine and nitroglycerine inhibited microsomal phosphodiesterase but activated the soluble form (V) The effect of adrenaline on the cyclic AMP content in vascular smooth muscle is probably the summed action of at least three different components, namely 1 3 and 5 in the following scheme. The same drug may therefore influence the cyclic AMP content simultaneously by different mechanisms The following components and the characteristics of these components have been observed in regulation of the cyclic AMP content of smooth muscle

1 An increase of the cyclic AMP content following adrenergic β receptor stimulation The effect was blocked by adrenergic β-receptor blocking agents and present in the Ca^{++} poor muscle too As there was no change of the phosphodiesterase activity after isoprenaline, and an activation of adenyl cyclase in smooth muscle homogenate (Robison et al. 1971 Triner et al 1971) and a stimulation of the adenyl cyclase activity was demonstrated *in the microsomal fraction (page 24)* it is probable that the effect was produced by an activation of adenyl cyclase. ACTH probably increased the cyclic AMP content in intestinal muscle by the same mechanism C8—CCK and prostaglandin E_1 probably also stimulated the adenyl cyclase activity in the sphincter Oddi

2 An increase of the cyclic AMP content combined with a Ca^{++} independent inhibition of the phosphodiesterase activity This action was produced by papaverine and nitroglycerine and eventually by diazoxide

Papaverine and nitroglycerine inhibited microsomal and other particle bound phosphodiesterases but activated the soluble form of the enzyme. There was no indication that these two drugs influenced the adenyl cyclase activity but diazoxide and hydralazine might influence the cyclic AMP content by other ways than via phosphodiesterase.

3 *A Ca^{++} dependent increase of the cyclic AMP content combined with a Ca^{++} dependent inhibition of the phosphodiesterase activity* This action was produced by a number of contracting drugs such as carbacholine, K^+ mepivacaine (intestinal smooth muscle) phenylephrine, histamine, K^+ (vascular smooth muscle) Whether the cyclic AMP increasing action was solely dependent on the inhibition of the phosphodiesterase activity or produced in some other way too has not been established

4 *A Ca^{++} independent decrease of the cyclic AMP content combined with an increased phosphodiesterase activity* was present after C8—CCK in the gallbladder and imidazole in intestinal smooth muscle. The effect was combined with contraction of the muscle.

5 *A Ca^{++} independent decrease of the cyclic AMP content associated with a decrease of the total phosphodiesterase activity* This action was present as an initial effect during isometric contraction of intestinal smooth muscle with carbacholine or K^+ and in vascular smooth muscle after α receptor stimulation by phenylephrine The effect was blocked by specific receptor antagonists In the Ca^{++} poor preparation the decrease was more sustained as it was in isotonically contracting vascular muscle after K^+ It seems probable that this effect was dependent on a reduction of the adenyl cyclase activity as carbacholine reduced this activity in the microsomal fraction (Fig 2) Alfa receptor stimulation was found to reduce the adenyl cyclase activity in a homogenate of rat uterus (Triner e t a l 1971) In heart muscle homogenate, acetylcholine reduced the adenyl cyclase activity an effect blocked by atropine (Murad e t a l 1962) A localized stimulation of the phosphodiesterase activity not detectable in the whole tissue homogenate might also be of importance however Amer (1971) has reported that adrenaline activated one form of phosphodiesterase (PDE II) in rabbit liver an effect blocked by phentolamine

6 *A Ca^{++} dependent decrease of the cyclic AMP content after α receptor stimulation combined with an activation of phosphodiesterase.*

The effect appeared with some delay after relaxation of the rabbit colon muscle and was combined with an increased ATP content and a reduced phosphorylase a activity These effects had a similarity with the opposite actions described under 3 Some other facts also indicate that the two cyclic AMP changes are contrary sides of the same mechanism The α receptor mediated relaxation in intestinal smooth muscle has been ascribed to both a direct effect on the smooth muscle cell (Brody and Diamond 1967 Bowman and Hall 1970) and a reduced activity of intramural cholinergic neurones in the intestinal wall Adrenaline has been shown to decrease the acetylcholine output of rabbit ileum by stimulation of adrenergic α receptors (Paton and Vizi 1969) Studies in this laboratory on "nerve free" and intact intestinal preparations from the rabbit indicate that the α induced relaxation is mediated both by a neuronal mechanism and a direct effect on smooth muscle (Wikberg personal commun.) The α receptor mediated relaxation might therefore be combined with a reduction of endogenously liberated acetylcholine. As cholinergic drugs increased the cyclic AMP content in intestinal muscle a reduced liberation of acetylcholine may lead to a decrease in the cyclic AMP content.

It is not probable that these are the only mechanisms by which drugs are able to influence the cyclic AMP content of smooth muscle preparations It can be speculated that the acetylcholine output from the intact intestine is dependent on the cyclic AMP content in the intramural neurones. Cyclic AMP facilitates the neural transmission and increases the output of acetylcholine (Singer and Goldberg 1970) It is therefore not unprobable that the reduction of the output of acetylcholine following adrenergic α receptor stimulation might be associated with a reduction of the cyclic AMP content in the neuronal tissue. This effect can, however not be detected under our experimental conditions, because of the small amount of neuronal tissue in relation to smooth muscles in rabbit colon muscle

Smooth muscle has been called "headache muscle and considering the mechanisms that influenced its cyclic AMP content this characterization seems not be unwarranted

Cyclic AMP and smooth muscle relaxation.

Several of the reported results indicate that cyclic AMP is a mediator of the relaxing action of adrenergic β-receptor agonists and some other drugs This hypothesis has been suggested by several investigators (Sutherland and Rall 1960 Mohme Lundholm 1963 Bueding e t a l. 1966 Lundholm e t a l. 1966 Levine e t a l. 1966 Dobbs and Robison 1968 Andersson and Mohme-Lundholm 1968 Moore e t a l 1968

Eggena et al 1968 Mitznegg et al 1970 Triner et al 1970 1971 Lamble 1970 Bowman and Hall 1970) The only investigators who on the basis of an eleborate experimental material have suggested that relaxation and the increase in the cyclic AMP content of smooth muscle are parallel but not causally related are Polacek and Daniel (1971)

The following discussion summarizes the most important findings that cyclic AMP mediates the relaxing action of some drugs or reduces the effect of contracting agents.

1 The increase in cyclic AMP content precedes the relaxing action of isoprenaline (II) and papaverine (V)

2 The degree of relaxation and increase in cyclic AMP are correlated after increasing doses of isoprenaline (II)

3 The potency of isoprenaline, adrenaline and noradrenaline to stimulate myometrial adenyl cyclase was of the same order as their relaxing effect on the intact uterine muscle (Dobbs and Robison 1968)

4 Adrenergic β-receptor blocking agents inhibited both the cyclic AMP increasing and the relaxing actions of catecholamines (II)

5 Isoprenaline stimulated the Ca^{++} binding of microsomal protein fractions from intestinal smooth muscle (IV) and stimulated the adenyl cyclase activity of these fractions. The effect of isoprenaline on the Ca^{++} binding was inhibited by an adrenergic β-receptor blocking agent. The fractions probably had functional similarity with the relaxing factor (sarcoplasmic reticulum) of skeletal muscle.

6 Exogenously added cyclic AMP had in some smooth muscle preparations, a more potent relaxing action than 5 AMP or other nucleotides. Cyclic AMP induced the same metabolic actions as hormones and drugs, which increased the cyclic AMP content and relaxed smooth muscle i e. activation of phosphorylase a and a Ca^{++} dependent reduction of the ATP content. Furthermore, it increased like isoprenaline and papaverine, the Ca^{++} binding of the microsomal fraction from intestinal smooth muscle

7 Several smooth muscle relaxing agents (papaverine, nitroglycerine, diazoxide theophylline) inhibited the phosphodiesterase activity (Kukovetz and Poch 1970 V) There was a dose response relationship between the phosphodiesterase inhibiting and relaxing action of papaverine (Poch 1971 Triner et al 1971) Papaverine and nitroglycerine very markedly inhibited the phosphodiesterase activity of the microsomal Ca^{++} binding protein fraction (V) Papaverine increased the Ca^{++} binding of this fraction too

8 Reduction of the cyclic AMP content through stimulation of th phosphodiesterase activity by C8—CCK prostaglandin E_2 (gallbladder) and imidazole (rabbit colon) was associated with contraction The rat of tension increase and maximal tension obtained by carbacholine and Ca^{++} (in the Ca^{++} poor rabbit colon) was increased by imidazole (VIII) In vascular smooth muscle, adrenergic α receptor stimulation was associated with a component leading to a reduction of the cyclic AMP content When isoprenaline simultaneously stimulated α and β receptors in the mesenteric artery blockade of the α receptor increased the relaxing action of the drug

9 In the Ca^{++} poor rabbit colon muscle addition of Ca^{++} contracted the muscle. An adrenergic β-receptor blocking agent prolonged and potentiated this contracting action (Andersson et al 1972 b)

These experiments show that there is a marked covariation between the relaxing and cyclic AMP increasing action of a number of drugs and that exogenous cyclic AMP mimics the relaxing and metabolic effects of these drugs. The findings are admittedly indirect and do not prove a causal relationship Those experiments showing a dissociation between relaxation and cyclic AMP formation are therefore of special interest Polacek, Bolan and Daniel (1971) reported that theophyllin diazoxide and papaverine in low concentrations relaxed the isotonically contracted rat uterus with minimal or no elevation of cyclic AMP levels. In judging these results the following facts are of interest to point out. The control muscles (rat uteri) showed rhythmic contractions the magnitude of maximal contraction or relaxation after the drugs seemed to differ very little from the spontaneous contracted or relaxed state. It is not stated whether the control muscle was frozen, and the cyclic AMP content determined during the relaxed or contracted state during its rhythmic activity The cyclic AMP level of smooth muscle may change within second (II) If the contractile state is influenced by the cyclic AMP level it must be of importance to make the comparison with a constant contracted or relaxed muscle as has been done in the present investigation on intestinal and vascular smooth muscle.

Another point is that some drugs can relax smooth muscle by more than one mechanism one of these does not involve cyclic AMP Adrenaline relaxed intestinal smooth muscle both through stimulation of α and β-receptors The α receptor mediated relaxation was not associated with any increase in the cyclic AMP level The L-isomer of the local anaesthetic agent mepivacaine (Åberg and Andersson 1972) had, in the rat portal vein, a cyclic AMP increasing and relaxing action which was reduced after adrenergic β-receptor blockade. The D isomer of the drug though a more potent relaxing agent, did not influence the cyclic

AMP level The action of the D isomer and part af the L form was probably dependent on a reduction of the permeability of the cell mem branes to Ca^{++} (Åberg and Andersson 1972) Papaverine posesses a local anaesthetic action (Grollman and Grollman 1970) On rat aorta its relaxing action was found to be diphasic an initial rapid relaxation was followed by a slower but sustained effect (Ericsson, personal com mun.) The objections raised by Polacek and Daniel are worth further studies but do not — in my opinion — seriously invalidate the hypothe sis that cyclic AMP is a mediator of the relaxing effects produced by some drugs especially β-receptor agonists

The mode of action of cyclic AMP in the contraction relaxation cycle.

If the contraction relaxation cycle is regulated by the free myoplasmic Ca^{++} concentration the relaxing action of those drugs which elevat the cyclic AMP level is likely to depend on a reduction of this Ca^{++} concentration. Free myoplasmic Ca^{++} can either be increased by a re lease of Ca^{++} from intracellular stores or by diffusion through the cell membrane from the extracellular fluid. According to van Bremen et a l (1972) and Keatinge (1972) noradrenaline releases Ca^{++} from int racellular stores in vascular smooth muscle, whereas depolarization of the membrane with K^+ increased the Ca^{++} diffusion through the cell membrane. Mepivacaine contracted intestinal and vascular smooth muscle even in a Ca^{++} free buffer solution, an effect probably depen dent on a release of Ca^{++} from intracellular stores since mepivacaine reduced the influx of ^{45}Ca (Åberg and Andersson 1972) The contrac ting action of adrenaline in mesenteric arteries was more sensitive to a reduction of the energy production than that of K^+ (Lundholm and Mohme Lundholm 1963) which may indicate that in the former case Ca^{++} had to be released from sites where the ion was bound by an ener gy dependent reaction

The release of Ca^{++} and transport of Ca^{++} across the cell membrane of smooth muscle is partly regulated by the action potentials of the cell membrane (Tomita 1970 Kuriyama 1970) In vascular smooth muscle, however there is probably a Na^+ contribution to the spikes (Keatinge 1972) In some vascular smooth muscle — such as rabbit pulmonary ar tery — the electromechanical coupling may take place via graded de polarization rather than repetitive spike electrogenesis (Somlyo and Somlyo 1968) Even in smooth muscle depolarized by KCl or K_2SO_4 some drugs can influence the contractile process without membrane po tential changes (Evans Schild and Thesleff 1958)

Relaxing action of cyclic AMP in relation to Ca^{++}

Drugs that change the cyclic AMP level of smooth muscle and exogenous applied cyclic AMP might relax smooth muscle either by increasing the Ca^{++} binding of intracellular stores or reducing the influx of Ca^{++} through the cell membrane In both cases the action might occur through an influence on the electrical activity of the membrane. Ca^{++} may have two functions at the membrane one as a current carrier and the other as a stabilizing agent (Tomita 1970 Kuriyama 1970) The calcium permeability of the plasma membrane, and other cell membranes is probably regulated by the amount of calcium bound to them the calcium permeability is increased in Ca^{++} free solution (Hurwitz, von Hagen and Joiner 1967 Somlyo and Somlyo 1968) Cyclic AMP might directly reduce the myoplasmic Ca^{++} concentration by accumulating Ca^{++} in stores and/or binding Ca^{++} to membranes Through the latter action the calcium permeability of the membranes surrounding the stores or the cell membrane itself may be reduced and the Ca^{++} concentration influenced.

Isoprenaline and cyclic AMP (IV) increased the Ca^{++} binding of the microsomal fractions isolated from intestinal muscle. The effect was maximal already within 60 sec, when the relaxing action also had reached a maximum The Ca^{++}-dependent ATP reducing action of isoprenaline — which probably was associated with the Ca^{++} binding effect — was present already within 10 sec, before the relaxation had started (I) ACTH and papaverine increased the Ca^{++} binding too The phosphodiesterase inhibiting action of papaverine was especially prominent in the microsomal fraction (V) Carbacholine, however which had a cyclic AMP-decreasing component in rabbit colon released Ca^{++} from the microsomal fraction preloaded with Ca^{++} (Nilsson and Andersson 1972)

The morphological localization of the Ca^{++} binding microsomal fractions from intestinal smooth muscle is not yet known. Carsten (1969) found that the corresponding fractions of uterine muscle were of vesicular nature. The existence of a longitudinal smooth sarcotubular system has been described by Lane (1965 1967) in intestinal muscle. Somlyo Devine and Somlyo (1972) described a sarcoplasmic reticulum in vascular smooth muscle in contact with surface vesicles" of the cell membrane. With reservations for histological differences between different kind of smooth muscle, the Ca^{++} binding fractions in rabbit colon may either be a part of or closely attached to the cell membrane. The different fractions may not have identical morphological localization, however

Of interest for this discussion are the results of Friedmann and Park (1968) who found that glucagon and cyclic AMP stimulated a probably energy dependent efflux of Ca^{++} and K^{+} through the cell membrane of liver cells In rabbit colon there probably exist a Ca^{++}-extrusion mechanism in the cell membrane too The efflux of ^{45}Ca from rabbit colon *preloaded with ^{45}Ca was however reduced by isoprenaline.* This drug did not influence the rate of the total Ca extrusion from the cold treated colon muscle. Cyclic AMP however reduced the total Ca extrusion in *this muscle (Djurv Andersson and Mohme Lundholm unpublished)* The effect of cyclic AMP on Ca efflux through the cell membrane in different tissues seems therefore not to be the same.

Cyclic AMP and electrical activity of the smooth muscle membrane.

The relaxing action of isoprenaline and cyclic AMP was reduced but still present in the K^{+}-depolarized rabbit taenia coli whereas the α receptor mediated relaxation was absent in the depolarized preparation (Andersson and Mohme Lundholm 1969) A part of the relaxing action of β-receptor stimulation is probably mediated by an action on the polarized cell membrane. The action of β-receptor *agonists and cyclic AMP* on the electrical activity of smooth muscle is therefore of interest.

The extensive studies of Bulbring and her coworkers (Bülbring e t a l 1970) *on the effect of adrenaline on the electrical activity of guinea* pig taenia coli are in this connection difficult to evaluate as the type of adrenergic receptor mediating the response has not been stated Since adrenaline in its lowest active concentration stimulated α receptors in this muscle (Andersson and Mohme Lundholm 1969) a pure α effect or a mixed α and β-response has probably been recorded in these studies

Somlyo Haeusler and Somlyo (1970) and Takagi, Takayanagi and Tomiyama (1971) found that isoprenaline and dibuturylcyclic AMP hyperpolarized smooth muscle membrane. Papaverine and nitroglycerine hyperpolarize the smooth muscle membrane too (Takagi e t a l 1971 Keatinge 1966) Glucagon and cyclic AMP are also reported to hyperpolarize the membrane of liver cells (Friedmann, Somlyo and Somlyo 19 1) and Purkinje s cells of rat cerebellum (Siggins e t a l 1971) C8—CCK, which reduced the cyclic AMP content in the gall bladder (VII) has been reported to increase the electrical activity of the muscle membrane (Golenhofen Loh and Lynen 1971) It seems therefore probable that cyclic AMP influences the electrical activity of the smooth muscle membrane *On the other hand Johansson e t a l* (1969) found that the relaxing action produced by stimulation of β-receptors in the rat portal vein was ass an increased spike discharge.

An increased extracellular Ca^{++}-concentration will stabilize the smooth muscle membrane, decrease its Na^{+} permeability and increase the K^{+} permeability A decreased Ca^{++} concentration will have the opposite effect (Tomita 1970 Kuriyama 1970) These actions are similar to that of adrenaline Bülbring and Kuriyama (1963) suggested that the action of catecholamines was dependent on a fixation of Ca^{++} to the membrane. These studies were done on the guinea pig taenia coli hence the action probably involves a stimulation of α receptors where an effect on the cell membrane is prominent (Andersson and Mohme Lundholm 1969) A mechanism involving binding of Ca^{++} to the cell membrane could explain the hyperpolarizing action of cyclic AMP on the smooth muscle membrane too The effect of separate α and β-receptor stimulation on the electrical activity and ionic permeability of the smooth muscle membrane need further studies for characterization, however

The depolarized smooth muscle was relaxed by β-stimulation and exogenous cyclic AMP An effect on the Ca^{++} binding mechanism independent of the membrane electrical activity may therefore be present It seems probable that Ca^{++} in this case is bound to intracellular stores An indirect support of this suggestion was that a previous β-receptor stimulation, but not an α stimulation, potentiated the contracting action of a subsequent dose of carbacholine in rabbit colon (VIII) This observations might indicate that the amount of Ca^{++} released by the contracting agent was increased by β-stimulation.

The Ca^{++} poor rabbit colon contracted by Ba^{++} was also relaxed by β-stimulation with utilization of ATP The effect was weaker however than in normal muscle (II) Batra and Daniel (1971 b) observed that the affinity of Ba^{++} for binding sites of the microsomal fraction from uterus was less than that of Ca^{++}

Relaxing action of cyclic AMP in relation to the carbohydrate metabolism.

The relaxing action of isoprenaline and cyclic AMP in rabbit colon was correlated with an increase in the phosphorylase a activity an effect independent of its ATP reducing action (I) A corresponding correlation was present after ACTH too in spite of its initial ATP increasing action (VI) Isoprenaline and cyclic AMP had no relaxing phosphorylase a activating or ATP reducing affects in the carbohydrate-poor muscle which still could be relaxed by adrenergic α receptor stimulation, however (I) These results indicate that the relaxing action of β-receptor stimulation and cyclic AMP was at least partly dependant on its stimulating effect on phosphorylase a activity and carbohydrate metabolism.

It seems not unlikely that the mechanism is associated with a stimulation of ATP synthesis. During the relaxing action of isoprenaline the ATP and CrP contents of rabbit colon were maximally reduced to about 50 % of the control values (I) In the Ca^{++} poor rabbit colon β-receptor stimulation or addition of cyclic AMP were in contrast associated with an increase in the ATP content about half as great as the reduction obtained in presence of Ca^{++} (II) The phosphorylase a activating effect of β-receptor stimulation and cyclic AMP therefore probably increase ATP synthesis and might thereby influence the ATP utilizing Ca^{++} binding process

In conclusion it is suggested that drugs which increase the cyclic AMP level and cyclic AMP itself may increase the Ca^{++} bindning to both intracellular stores and the cell membrane. Cyclic AMP will probably not stimulate the Ca^{++} transport across the cell membrane, however At least three different Ca^{++} binding microsomal fractions with phosphodiesterase activities were isolated from rabbit colon These fractions might correspond to different binding sites for Ca^{++} in the smooth muscle cell

Cyclic AMP changes and contraction of smooth muscle.

If an increase in the cyclic AMP level of *smooth muscle induced a re*laxing action it is probable that a decrease in this level was associated with a raised tension This was the case when the cyclic AMP level was decreased by hormones or substances which activated phosphodiesterase such as C8—CCK PGE_2 and imidazole (VII and VIII)

The influence of other contracting drugs on the cyclic AMP level was more complex For example carbacholine and K^+ (in rabbit colon) and α receptor stimulation and K^+ (in mesenteric artery) had both a decreasing and an increasing component on the cyclic AMP level (VIII and III) The eventual role of a decreased cyclic AMP level in the contracting action of these drugs is therefore much more controversial The following fact speaks however in favor of a connection between contraction and a decreased cyclic AMP level

The cyclic AMP level was reduced at a very early stage and before the tension had started to develop The decreasing effect was mediated by a stimulation of cholinergic or adrenergic receptors since the action *was blocked by atropine (rabbit colon) or dibenamine (mesenteric arte*ry) even in the Ca^{++} poor preparation (VIII and III) Carbacholine released Ca^{++} from a microsomal fraction preloaded with Ca^{++} (Nilsson and Andersson, unpublished) and reduced its cyclic AMP formation.

Speaking against a role of cyclic AMP in contraction is the fact that later during the tension development and very late during isotonic shortening the cyclic AMP level increased to the same level produced by relaxing drugs Two explanations may be considered 1 Th decrease in cyclic AMP was produced by receptor stimulation but was a parallel phenomenon not causally related to the contractile process. 2. The cyclic AMP regulating enzymes are compartmentilized in the smooth muscle cell There is a constant decrease at some important Ca^{++} binding site but an increase of the level in the cytoplasm conceals the local decrease. Evaluating these two alternatives it is hard to accept the first, from which it follows that an increase in the cyclic AMP level relaxed the muscle whereas a decrease had no influence on the contractile process For the second alternative speaks the fact that there are indications of compartmentilization of the cyclic AMP regulating enzymes in smooth muscle.

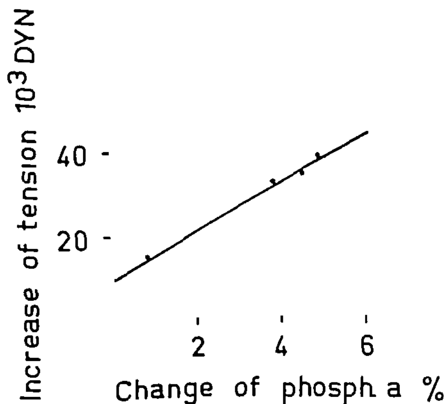


Fig 4

R l t onsh p between ncrease f tens on nd phosphorylase a tivation in colon muscl 5 min after treatment with 1 7×10⁻ g/ml carbach l ne.

Compartmentilization of cyclic AMP in smooth muscle.

Papaverine stimulated the phosphodiesterase of cytoplasm but inhibited the structurally bound enzyme (V) It increased the phosphorylase a activity to some degree, but there was a negative correlation between the relaxing and phosphorylase activating effects of papaverine. At 100 % relaxation the phosphorylase a activity was not increased (V) Phosphorylase is a soluble enzyme and the phosphorylase a activity will probably reflect changes of the cytoplasmic cyclic AMP level The rise in cyclic AMP level after carbacholine was proportional to th in crease in tension (VIII) and a corresponding relationship was found between the increase in phosphorylase a and tension after carbacholine (fig 4) It is therefore probable that carbacholine had increased the level of cytoplasmic cyclic AMP In the Ca^{++} poor preparation the decreasing action of carbacholine and a stimulation on the total cyclic AMP level was present at a time when the level was raised in the normal muscle (VIII) The decreasing and increasing components of carbacholine on the cyclic AMP level were therefore probably present simultaneously and the decreasing component might be localized to some other compartments than the cytoplasm of the cell

To test this hypothesis the action of carbacholine on cyclic AMP formation of the Ca^{++}-binding microsomal fraction was studied As can b seen from fig 2 carbacholine reduced the cyclic AMP formation whereas isoprenaline increased it These effects are similar to those of β-receptor stimulation and acetylcholine stimulation on the adenyl cyclase activity of a homogenate from dog heart (Murad e t a l 1969) although the actions of the drugs on the contractile process was the reverse from that of smooth muscle. There is thus some indications that the decreasing and increasing actions of carbacholine on the cyclic AMP formation is localized within different compartments of the smooth muscle cell

The cell membrane is, however permeable to cyclic AMP to a certain degree the nucleotide can thus leave the cell through diffusion (Kuo and de Renzo 1969 Robinson e t a l 1971 Andersson e t a l 1972 c) It is probable that a raised cyclic AMP level in one compartment will to some degree, influence the level of another If this suggestion is right the increase in tension after a contracting drug will diminish when the total cyclic AMP level of the cell begins to increase When rabbit colon or bovine mesenteric artery contracted the maximal tension had not been reached before the cyclic AMP level increased why cyclic AMP may reduce development of the maximal tension The increasing action of imidazole on the contracting effect of carbacholine and Ca^{++} can be explained by such a mechanism (VIII) In tests on

notonically contracting mesenteric arteries the rise in cyclic AMP was very slow From fig 3 is seen that there was some reduction in the degree of shortening after 15 min when total cyclic AMP content had increased in comparison with the values after 5 min

The vascular escape phenomenon i e the secondary decrease in resistance by a vasoconstrictive agent may have the same mechanism Henrich, Biester and Lutz (1972) found that adrenergic β-receptor blocking agents did not influence this phenomenon This is in agreement with the findings that the cyclic AMP increasing action of phenylephrine and adrenaline in mesenteric artery was not inhibited by β-receptor blocking drugs (III)

Atropine blocked the cyclic AMP decreasing effect of K^+ in rabbit colon but not the contraction produced by the ion (page 29) Both actions of carbacholine were blocked by atropine (VIII) Paton and Vizi (1969) found that K^+ released acetylcholine from intramural neurones in rabbit intestine but that the contractile effect of the ion was only partly dependent on this action. The decreasing action of K^+ on the cyclic AMP level might therefore depend on a release of acetylcholine. Van Bremen e t a l (1972) reported that K^+ in vascular smooth muscle increased the influx of extracellular Ca^{++} It might be concluded from these facts that cyclic AMP at least in rabbit colon influences the release and uptake of Ca^{++} in intracellular stores, but has less effect on the flux of Ca^{++} through the cell membrane in agreement with findings reported on pag 40 Whether the cyclic AMP reducing effect of K^+ in mesenteric arteries (III) depends on a release of noradrenaline from adrenergic neurones and can be blocked by α receptor blocking drugs remains to b established

Functional significanse of cyclic AMP increase during contraction.

If it is supposed that the cyclic AMP-decreasing action of carbacholine and α receptor stimulation is mediating the contractile action of these drugs the functional significance of the cyclic AMP increasing action can be discussed Contraction of smooth muscle is associated with an increased utilization of ATP (Beviz e t a l 1965 1969 Andersson Lundholm and Mohme—Lundholm 1971) One effect of drugs increasing the cyclic AMP level and phosphorylase a activity is to increas the ATP synthesis during contraction In skeletal muscle, the increase of phosphorylase a activity during contraction is produced by Ca^{++} activation of phosphorylase b kinase cyclic AMP is not involved (Walsh e t a l. 1970) In smooth muscle, the corresponding process is activated by Ca^{++} too but cyclic AMP is probably mediating th effect of Ca^{++} (VIII)

Both stimulation of β-receptors and exogenous cyclic AMP potentiated the subsequent contraction of carbacholine in rabbit colon (VIII). Cyclic AMP when acting on the Ca^{++} binding sites of smooth muscle might therefore load up the stores of calcium. The observations of Bartelstone et al (1967) that theophylline, under some conditions potentiated the contracting effect of noradrenaline on rat aorta may have the same explanation. This kind of negative feed back between cyclic AMP and Ca^{++} might have two functions. First, Ca^{++} released from the stores will be rapidly pumped back so that a contractile impulse is not prolonged, i.e. the myogenic activity can be continuously and rapidly regulated. Secondly, through a previous β-receptor stimulation, the contractile action of a drug or a membrane depolarization might be potentiated, an action which might partly explain the positive inotropic effect of β-receptor stimulation in the heart (Entman et al 1969).

The role of cyclic AMP in the contractile process of smooth muscle is admittedly more complicated and difficult to evaluate than its action on smooth muscle relaxation. On the other hand, it seems unlikely that the marked cyclic AMP changes during contraction are without functional significance for the contractile process.

Summary

The connection between changes of the cyclic AMP level and relaxation, contraction and metabolic processes in smooth muscle has been studied, as well as the role of Ca^{++} in these relationships. The following results have been found

1 In rabbit colon muscle relaxation was induced both by adrenergic α and β-receptor stimulation. The adrenergic α receptor mediated relaxation was not initially associated with metabolic changes, but after complete relaxation, the cyclic AMP level phosphorylase a activity and glycogenolytic metabolites were reduced whereas the content of ATP and CrP was increased. The relaxation mediated by adrenergic β-receptors was preceded by an increase of the cyclic AMP level a stimulation of phosphorylase a activity and an increase in the content of hexosephosphates, but the content of ATP and CrP was reduced In bovine mesenteric artery stimulation of adrenergic α receptors produced a contraction whereas β-receptor stimulation caused a relaxation. The relaxing effect was associated with metabolic changes similar to those of colon muscle. The contracting effect was initially associated with a decreased cyclic AMP level When the tension was maximal the content of cyclic AMP was increased as was the phosphorylase a activity

2 The adrenergic β-receptor mediated relaxation of rabbit colon and bovine mesenteric artery was preceded by an increase of the cyclic AMP content and a stimulation of the phosphorylase a activity but a decreas of ATP and CrP contents These metabolic changes were further accentuated when the relaxation was complete. The reduction of high energy phosphate compounds was reversed to an increase after 10 min however The degree of relaxation was correlated to the increase in cyclic AMP to the reduction of the ATP-content and to the increase in the phosphorylase a activity The relaxing and metabolic effects were blocked by an adrenergic β-blocking agent like the relaxation.

3 Exogenous cyclic AMP relaxed rabbit colon muscle and bovine mesenteric artery and produced the same metabolic changes as adrenergic β-receptor stimulation. Adenosine 5' monophosphate and adenosine relaxed the colon muscle and moderately increased the cyclic AMP level too 5' AMP reduced the level of ATP in colon muscle

4 Rabbit colon muscle was relaxed by papaverine nitroglycerine, ACTH and PGE_1 There was a quantitative correlation and a coordination in time between relaxation and increase of cyclic AMP content after these substances. Diazoxide and hydralazine relaxed bovine me-

senteric artery this effect was associated with an increased cyclic AMP level In sphincter Oddi from cat the C-terminal octapeptide of cholecystokinin and PGE_2 increased the cyclic AMP level and relaxed th sphincter The relaxing effect of papaverine, nitroglycerine, diazoxide and hydralazine was also associated with a reduction of the content of ATP and a moderate stimulating effect on the phosphorylase a activity

5 The cyclic AMP increasing effect of papaverine and nitroglycerine was associated with a reduction of the phosphodiesterase activity Papaverine inhibited phosphodiesterase from colon muscle *in vitro* On a fractionated homogenate, phosphodiesterase localized in the microsomes was markedly inhibited whereas that localized in cytoplasm was stimulated Nitroglycerine reduced the phosphodiesterase activity of a colon homogenate markedly and the effect on the different cell fractions was similar to that of papaverine

6 A reduction of the Ca content of colon muscle or mesenteric artery *was associated with reduced contents of cyclic AMP hexosephos*phates and high energy phosphate compounds The phosphorylase a activity was also reduced On addition of Ca^{++} or Ba^{++} to the Ca^{++} deficient muscle the level of the metabolic parameters studied was changed to that of normal muscles

7 In Ca^{++} deficient muscle the metabolic changes of adrenergic β-receptor stimulation was as in normal muscle, with the exception of the initial ATP reduction which was not present The metabolic changes of adrenergic α receptor stimulation in rabbit colon were blocked in the Ca^{++} poor muscle. Adrenergic α receptor stimulation of Ca^{++} deficient mesenteric arteries was associated with a reduction of th cyclic AMP content. In these preparations however there was no secondary rise of the cyclic AMP level

8 Microsomal fractions which bound Ca^{++} in the presence of ATP were isolated from colon muscle The Ca^{++} binding process was stimulated by cyclic AMP isoprenaline and papaverine. Carbacholine released Ca^{++} from these fractions however The cyclic AMP regulating enzymes adenyl cyclase and phosphodiesterase, were present in the microsomal fractions. The adenyl cyclase activity was stimulated by isoprenaline but inhibited by carbacholine

9 Contraction induced by imidazole C8—CCK and PGE_2 in rabbit colon muscle and gallbladder of guinea pig was preceded by a stimulation of the phosphodiesterase activity and a decrease of the cyclic AMP level

10 The contraction induced by carbacholine and potassium ions in rabbit colon was preceded by a decrease of cyclic AMP content too but when the tension development was maximal the cyclic AMP content was

increased instead The cyclic AMP level of mesenteric artery was studied after isometric and isotonic contraction with potassium ions. The reduction of cyclic AMP content was more prolonged during isotonic contraction than during isometric contraction The reduction of the ATP level was more pronounced during isometric than isotonic contraction.

11 The cyclic AMP increasing effect of contracting agents was associated with a reduction of the phosphodiesterase activity These effects were Ca^{++} dependent. It was found that Ca ions reduced the phosphodiesterase activity of muscle homogenate of rabbit colon at concentrations which induce a contraction.

12 Cyclic AMP is suggested to be a regulator substance for the contraction relaxation cycle of smooth muscles Increase in the tissue level of cyclic AMP would then stimulate an energy requiring Ca^{++} binding mechanism which in turn, probably leads to a decreased concentration of myoplasmic Ca^{++} and subsequent relaxation A reduction of the cyclic AMP level caused by contracting agent either through a stimulation of phosphodiesterase or a reduction of the adenyl cyclase activity probably leads to a release of bound Ca which activated the contracting proteins. The contraction was potentiated by a previous β-receptor stimulation probably because more Ca^{++} had been bound and could be released. Both relaxation and contraction were energy requiring processes. The glycogenolysis was stimulated as a parallel phenomenon to produce energy for these processes. An increased cyclic AMP level and stimulation of phosphorylase a activity was obtained by β-receptor stimulation and secondary to the tension development of contracting agents. The dual action of contracting agents on the cyclic AMP level is assumed to reflect a compartmentilization of the cyclic AMP system in the smooth muscle cell. One fraction located to the microsomes influences Ca^{++} binding and release another cytoplasmic fraction regulates the phosphorylase a activity and glycogenolysis.

senteric artery this effect was associated with an increased cyclic AMP level In sphincter Oddi from cat the C terminal octapeptide of cholecystokinin and PGE_1 increased the cyclic AMP level and relaxed th sphincter The relaxing effect of papaverine, nitroglycerine, diazoxide and hydralazine was also associat~d with a reduction of the content of ATP and a moderate stimulating effect on the phosphorylase a activity

5 The cyclic AMP increasing effect of papaverine and nitroglycerine was associated with a reduction of the phosphodiesterase activity Papaverine inhibited phosphodiesterase from colon muscle *in vitro* On a fractionated homogenate, phosphodiesterase localized in the microsomes was markedly inhibited whereas that localized in cytoplasm was stimulated Nitroglycerine reduced the phosphodiesterase activity of a colon homogenate markedly and the effect on the different cell fractions was similar to that of papaverine.

6 A reduction of the Ca content of colon muscle or mesenteric artery was associated with reduced contents of cyclic AMP hexosephosphates and high energy phosphate compounds The phosphorylase a activity was also reduced On addition of Ca^{++} or Ba^{++} to the Ca^{++} deficient muscle the level of the metabolic parameters studied was changed to that of normal muscles

7 In Ca^{++} deficient muscle the metabolic changes of adrenergic β-receptor stimulation was as in normal muscle with the exception of the initial ATP reduction which was not present The metabolic changes of adrenergic α receptor stimulation in rabbit colon were blocked in the Ca^{++} poor muscle Adrenergic α receptor stimulation of Ca^{++} deficient mesenteric arteries was associated with a reduction of th cyclic AMP content In these preparations however there was no secondary rise of the cyclic AMP level

8 Microsomal fractions which bound Ca^{++} in the presence of ATP were isolated from colon muscle. The Ca^{++} binding process was stimulated by cyclic AMP isoprenalin and papaverine. Carbacholine released Ca^{++} from these fractions, however The cyclic AMP regulating enzymes adenyl cyclase and phosphodiesterase were present in the microsomal fractions. The adenyl cyclase activity was stimulated by isoprenaline but inhibited by carbacholine

9 Contraction induced by imidazole C8—CCK and PGE_2 in rabbit colon muscle and gallbladder of guinea pig was preceded by a stimulation of the phosphodiesterase activity and a decrease of the cyclic AMP level

10 The contraction induced by carbacholine and potassium ions in rabbit colon was preceded by a decrease of cyclic AMP content too but when the tension development was maximal the cyclic AMP content was

References

AHLQUIST R.P and B LEVY J Pharmacol exp Ther 1959 127 146

AMER, M.S Clin Res. 1969 17 590

AMER, M.S In Abstract, Physiology and Pharmacology f Cycl c AMP Milan, 1971 8

ANDERSSON R.G G and E. MOHME-LUNDHOLM Brit. J Pharmacol. 1965 34 204 p

ANDERSSON R. nd E. MOHME-LUNDHOLM Acta physiol sc nd. 1969 a, 77 87_

ANDERSSON R. and E. MOHME LUNDHOLM Proc 4 th Int. Congr Pharmacol. Basel, 1959 b (Schwabe & Co Publ. 1970)

ANDERSSON R, N.P BERGH and N SVEDMYR. Scand J resp Dis. 1972, 53 125

ANDERSSON R, L. LUNDHOLM and E. MOHME-LUNDHOLM Proc. Symposium Physiol. Pharmacol. Vasc Neuroeffector System S S Ka ger Basel, 1971 902.

ANDERSSON R L. LUNDHOLM nd E. MOHME-LUNDHOLM. I Abstra t Physiology and Pharmacol gy of Cyclic AMP Milan, 1971 b, 63

ANDERSSON, K.E., R. ANDERSSON P HEDNER and C.G.A PERSSON Lif Sci. 197 a, 11 725

ANDERSSON K.E., R ANDERSSON P HEDNER and C.G.A. PERSSON Acta Pharmacol (Kbh) 1972 b, 31 suppl I 44

ANDERSSON R., L HARTHON M HEDSTROM nd L. LUNDHOLM Lif Sci 1972 (In press)

ANDERSSON,R L. LUNDHOLM E MOHME-LUNDHOLM nd K NILSSON In Advances i cyclic nucleotide resea ch vol. I (Eds Greenga d, R. Paoletti, G.A Rob son) Raven Press N Y 1972 d, 499

APPLEMAN M M and R G KEMP Biochem. biophys. Res. Commun 1966 24 564

ARIENS E J Ann N Y Ac d. Sci. 1967 139 606.

AXELSSON J E BUEDING nd E. BULBRING J Phys l 1961 156, 357

BARTELSTONE, H.J P A NASMYTH and J.M. TELEFORD J Physiol. 1967 188, 159

BATRA S.C and E.E. DANIEL. Comp. Biochem. Physiol 1971 a, 38 A 785

BATRA S.C nd E E. DANIEL Comp Biochem. Physiol. 1971 b, 38 A, 369

BAUER H P J GOODFORD nd J HUBER. J Physiol. 1965, 176, 163

BEAVO J A J G HARDMAN and E.W SUTHERLAND J Biol. Chem. 1970, 45 3649

BERGMEYER, H V and H. KLOTCH. I Methods f enzymati analyses (ed. H V Bergmeyer) Verlag Chemie, Weinheim, 1962, 131

Acknowledgements

To my respected teacher Professor Lennart Lundholm I wish to express my cordial gratitude. His wide knowledge and jovial personality have created the inspiring atmosphere, which was necessary for the performance of this work. Without his never failing trust and encouragement the present work would not have been possible.

My sincere gratitudes are also due to my collaborators Doc Ella Mohme Lundholm, Doc. Nils Svedmyr Doc Pavo Hedner and Drs Karin Nilsson, Karl Erik Andersson and Nandor Vamos.

My warm thanks are due to Miss Gun Abrahamsson, Miss Marianne Borg and Mrs Ingrid Salomonsson Mrs Anna Karin Falk and Mr Sverker Borjesson for their skilful technical assistance, and to Mrs Kerstin Nilsson for efficient secretarial aid.

For kindly revising the English text, I wish to thank Ph.D Roger Cunningham.

My deep thanks also to my wife Elisabet and my sons Fredrik and Henrik for constant encouragement patience and support.

The studies were supported by grants from the Swedish State Medical Research Council (B 70—14X—2080 and B 70—14X—101) the Magnus Bergvall Foundation The Majen and Gustav Lindgren Foundation the Swedish Society for Medical Research and the Medical Faculty School of Medicine, Linkoping

ENTMAN M.L., C L. LEVY and S E. EPSTEIN Ci Res 1959 2 3, 429

ERIKSSON E.E. Acta pharmacol. (Kbh) 1972, 3 1 45

VON EULER, U.S Clin Pharmac I Therap 196 9 28.

EVANS, D H L., H O SCHILD and S THESLEFF J Physiol. 1958, 1 4 3, 4 4

FEINSTEIN M.B J gen. physiol 1964 4 8 35

FILO R.S D BOHR and J C. RUEGG Scienc 1965, 1 4 7 1551

FRIEDMAN N and C.R PARK. Proc. N t Acad. Sci. 1968, 6 1 504

FRIEDMAN N A V SOMLYO and AP SOMLYO Sci nce 1971 1 7 1 400

FREUND J.E. Mathematichl Stat st cs. P entice H ll N Y 1952.

FURCHGOTT R F J Pharmacol p Ther 1954 1 1 1 765

FURCHGOTT R.F I Ciba Foundation Sympo ium on drene gi mechanisms (J.R Vane, G E.W W l tenh lme nd M.O O'Connor eds.) Churchill, London 1960, 16.

GEBERT G H. NGUYEN DUONG W SCHNIZER, P KONOLD F HILLEN BRAND H. YABU and K. BRECHT Arztl Forsch 1969 3, 391

GILMORE N J.E. VANE and J H WYLLIE N ture, (Lond) 1968, 2 1 8 1135

GOLENHOFEN K., D V LOH a d F.A LYNEN Experientia, 1971 7 650

GRAHAM J M nd W R KEATINGE. In V scula Smooth Mus le (E. Betz ed) Springer Berlin, 197_ 61

GROLLMAN A nd E F GROLLMAN Ph rmacology nd Therapeutics. Lea & F biger Philadelphia, 1970

HASHIMOTO K S KUMAKURA nd I TANEMURA. Arzneimett I F rsch 1964 1 4 12_

HASSELBACH W Progr b ophy Chem 1964 1 4 169

HAUGAARD N C S HAUGAARD N H LEE nd R.S HORN Fed. Proc 1969 2 8, 1637

HENRICH, H J BIESTER nd J CATZ I V scular Smooth Muscle (E. Betz ed.) Springer Berlin, 197_ 49

HESS, M L F M BRIGGS E SHINEBOURNE and J HAMER, Nat (Lond.) 1968 u. 9

HINKE, J A M I Muscle (W P L E E Daniel C.M. kay nd G Monckton ed) Pergamon Press, London, 1965, 269

HOHORTS H J I Metod of enzymatic analysi (H.V Bergmeyer ed.) Verlag Chemie Weinheim 1962, 134.

HURWITZ. L.D F FITZPATRICK, E J LANDON and G DEBBAS. Fed. P oc 19_ 35

HURWITZ L P D JOINER and S von HAGEN Proc. Soc exp. Biol Med. 1967 1 3 315

HURWITZ L P D JOINER and S von HAGEN Amer J Physi l 1957 1 3, 1299

JENKINSON D H. nd J.K.M MORTON N ture (Lond) 1965 2 0 5 505

AB Östgötatryck Linköping 1972

ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 383

Adrenergic, cholecystokinetic and morphine-induced effects on extra-hepatic biliary motility

By

Carl G. A. Persson

LUND 1972

LINKÖPING UNIVERSITY MEDICAL DISSERTATIONS

No 7

Editor The Dean of the Medical Faculty

[1] EVA BRITA MÜLLER *Studies in Diabetic Pregnancy* 1970.

[2] LARS-OLOF LAMKE *Evaporative Water Loss from Normal and Burnt Skin.* 1971

[3] ARNE TÄRNVIK *Influence of Red Cell Membranes on Phytohaemagglutinin-induced Lymphocyte Stimulation* 1971

4 ANDERS FORKMAN *Studies on Carbonic Anhydrase in Neisseria* 1972.

[5] GUNNAR ÅBERG *Studies on Mepivacaine and its Optically Active Isomers with Special References to Vasoactive Properties.* 1972.

6 BALZAR ALEXANDERSON: *On Interindividual Variability in Plasma Levels of Nortriptyline and Desmethylimipramine in Man A Pharmacokinetic and Genetic Study* 1972.

7 ROLF G G ANDERSSON *Cyclic AMP and Calcium Ions in Mechanical and Metabolic Responses of Smooth Muscles Influence of some Hormones and Drugs.* 1972

ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 383

Adrenergic, cholecystokinetic and morphine-induced effects on extra-hepatic biliary motility

By

Carl G.A. Persson

LUND 1972

ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 383

From AB Draco* Research and Development Laboratories,
Fack, S-221 01 Lund, Sweden.

Adrenergic, cholecystokinetic and morphine-induced effects on extra-hepatic biliary motility

By
Carl G.A. Persson

LUND 1972

*Subsidiary to AB Astra, Sweden.

Rahms Boktryckeri AB Lund 1972

Table of contents

In this survey the following papers will be discussed.

I *Adrenoceptors in the cat choledochoduodenal junction studied in situ.* G Liedberg and C.G.A. Persson Br J Pharmacol. *39* 619—626, 1970

II *Adrenoceptor functions in the cat choledochoduodenal junction in vitro* C.G.A Persson. Br J Pharmacol *42* 447—461 1971

III *Excitatory effect of tetrodotoxin on an isolated smooth muscle organ* C.G.A. Persson J Pharm. Pharmacol. *23* 986—987 1971

IV *The action of morphine on the cat choledochoduodenal tract* C.G.A Persson. Acta pharmacol (Kbh) *30* 321—329 1971

V *Resistance to flow through the pancreatic duct by the isolated cat sphincter of Oddi* C.G.A Persson Experientia, *28* 276—278 1972

VI *Adrenoceptors in the gallbladder* C.G.A. Persson Acta pharmacol. (Kbh) *31* 177—188 1972.

VII *Effect of morphine cholecystokinin and sympathomimetics on the sphincter of Oddi and intramural pressure in cat duodenum* C.G.A Persson and M Ekman Scand J Gastroent. 7 345—351 1972

VIII *Effect of cholecystokinin on the level of cyclic AMP and on mechanical activity in the isolated sphincter of Oddi* K.E. Andersson R. Andersson, P Hedner and C.G.A Persson Life Sci *11* 723—732, 1972.

IX *Dual effects on the sphincter of Oddi and gallbladder induced by stimulation of the right great splanchnic nerve* C.G.A. Persson. Acta Physiol. Scand In press.

The papers will be referred to in the text by their roman numerals.

Introduction

A. Historical notes

Bergh (1942) and Dowdy Jr (1969) have reviewed the early history of our knowledge of the gallbladder and bile ducts. In 1543 Vesalius demonstrated that the common bile duct entered the duodenum not the stomach, as had been believed previously A muscular sphincter at the termination of the common bile duct was described by Glisson (1681), and Vater (1720) reported that the mucosa was elevated at the orifice in the duodenum The elevation is known as the papilla of Vater (Fig. 1) The choledochoduodenal sphincter in the cat was first described by Gage (1879) Oddi (1887) rediscovered 'Glisson's sphincter' in several species and measured the resistance of the sphincter It has since borne the name sphincter of Oddi Careful anatomical investigations by Boyden and associates (Eichhorn and

Fig 1

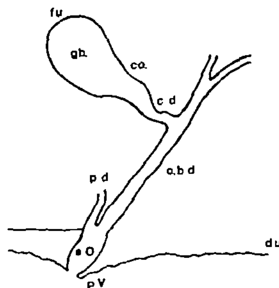


Fig 1
Shematic drawing of the anatomy of the xtra-hepatic biliary tract
f =fundus; gb=gallbladder co=collum, cd=cystic duct, bd=common bile duct; pd=pancreatic duct; sO=sphincter of Oddi; pV =papilla of Vater du=duodenal wall

Boyden, 1955 Boyden 1957 a, Boyden 1957 b) have revealed that the sphincter of Oddi is composed of muscular tissue that differs from duodenal muscle in structure and embryological development

Along with the increasing anatomical knowledge studies concerning the physiology and pharmacology of the biliary tract proceeded rapidly In 1934 Ivy reviewed over 500 papers on this subject. Current views on biliary function and motility were presented by Hallenbeck (1967)

B. Anatomical structures of importance in biliary motility

The gallbladder and the sphincter of Oddi are the most important structures in biliary dynamics (Fig. 1) Neither in the Lütkens sphincter in the collum part of the gallbladder nor in the valved cystic duct the valves of Heister has a true sphincteric function been established (Rothman 1965) However experimental data showing pressure differences between the gallbladder and the common bile duct may suggest that these parts of the biliary tract have a physiological sphincter function (Benzi and Frigo 1963 Doyle and Farrar 1969) There are also indications that the common bile duct can exhibit peristaltic movements and may thus play a role in biliary motility (Daniels, Mc Glone Job and Sawyer 1961 Hauge and Mark 1965 Ludwick 1966 Watts and Dunphy 1966 Golenhofen 1971)

It is generally agreed that the sphincter of Oddi can be influenced by the state of contraction of the surrounding duodenum This influence may manifest itself especially when the duodenal tone is increased e g. by the action of morphine (papers IV and VII) The possibility of a duodenal in fluence does not necessarily conflict with the concept of an independent sphincter mechanism operating under most physiological conditions (Lueth 1931 Bergh and Layne 1940 Magee 1946 Hallenbeck 1967)

C. Regulation of biliary dynamics

The most important mechanical effects of biliary smooth muscle seem to be produced by the hormone cholecystokinin (CCK) CCK has been demon strated to relax the sphincter of Oddi and contract and evacuate the gall bladder (Ivy and Oldberg 1928 Sandblom Voegtlin and Ivy 1935) Little is known about the mechanisms involved Furthermore the mechanical effect of CCK on the duodenal tissue surrounding the sphincter of Oddi has not been settled The mechanism of action of CCK and the duodenal effect are discussed in papers II VII and VIII

The role of the autonomic nervous system in biliary dynamics has not been established. In papers I II V VI and IX, special attention has been devoted to the possible role of the adrenergic nervous system in the control of biliary

motility In the evaluation of adrenergic effects it should be of value to know the distribution and functions of adrenoceptors in biliary smooth muscle. The presence of adrenoceptors in gallbladder smooth muscle has not been established previously In the isolated sphincter of Oddi the α receptor has been described but there is still some doubt about the existence of a β-receptor (Crema and Berté 1963) Concerning the α-receptor function and the effect of adrenalin and noradrenalin there seems to be no agree ment between *in vitro* and *in situ* findings (Crema and Berté 1963 Benzi Berté Crema and Frigo 1964) The adrenoceptor functions in the gallbladder and the sphincter of Oddi are demonstrated in papers I II and VI where also *in vitro* findings are correlated with results obtained *in situ*. In addition effects on the biliary tract induced by the stimulation *in situ* of the adrenergic nerve supply are reported in paper IX. The nervous effects are explained in terms of adrenoceptor distribution and function and are correlated to *in vitro* effects.

It is generally agreed that the sphincter of Oddi can function autonomously and can exhibit spontaneous activity (Bergh 1942, Magee 1946, Crema and Berté 1963 Wyatt 1967) It is not known whether the sphincter activity is myogenic or nervous in origin Furthermore, it is controversial whether the sphincter activity promotes or inhibits the flow of bile into the duodenum (Watts and Dunphy 1966 Toouli and Watts 1972) Another question which so far has not been studied, is how the sphincter of Oddi affects flow resistance through the main pancreatic duct. These aspects of sphincter function were studied in papers II and V

Morphine has important effects on biliary dynamics. It increases the intra ductal pressure by a spasmogenic effect at the choledochoduodenal junction. It is not known, however whether this action is due to effects on the in testine only or on the sphincter of Oddi as well (Hallenbeck 1967 Daniel 1968) It is currently under discussion whether the spasmogenic effect of morphine is nerve-mediated or a direct effect on smooth muscle (Daniel 1968) A third possibility is that morphine acts indirectly through release of a spasmogenic non-nervous agent (cf Burks and Long 1967) The finding by Crema, Benzi Frigo and Berté (1965) that the morphine-spasm was not blocked by agents known to block peripheral effects of the autonomic ner vous system, plus the surprising observation by the same authors that mor phine was less effective intraarterially than intravenously at the choledochoduodenal junction seemed to need confirmation as was pointed out by Daniel (1968) The effects of morphine on choledochoduodenal tissues were studied in papers IV and VII.

D Aims of the present investigation

To summarize the aims of the present investigation were.

1 To study the mechanism of action of CCK on biliary smooth muscle and the effect of CCK on the part of duodenum close to the sphincter of Oddi.

2. To study adrenergic effects on biliary motility

3 To study the nature and function of the spontaneous activity of the sphincter of Oddi

4 To study the effect of morphine on the choledochoduodenal junction.

Experimental Approach

A. Animal

In the cat, the anatomy of the biliary tract has been thoroughly demonstrated by Boyden (1957 a) who also showed that the cat sphincter of Oddi had important resemblances to man e.g. in both species the main pancreatic duct opens into the duodenum through the sphincter of Oddi. The innervation of the cat biliary tract was described by Hirt (1934) The adrenergic nerves were further surveyed by use of the fluorescence method of Falck and Hillarp (Baumgarten and Lange 1969) The cat has been used in a number of investigations dealing with the pharmacology and physiology of biliary dynamics (e g. Ivy 1934 Granser Hertting, Rissel and Wewalka 1956 Benzi Berté, Crema and Frigo 1964 Pallin and Skoglund 1964 Hallenbeck 1967) This existent knowledge of the cat biliary tract contributed to the choice of this animal for the various investigations presented in papers I—IX.

B. Methods

The various methods used for recording the activity of the gallbladder the sphincter of Oddi and the duodenum are described in detail in the special papers. Conventional techniques were applied for measuring isometric tension changes in the isolated smooth muscle tissues (papers II III IV V VI and VIII) Intraluminal pressure changes in the gallbladder and the duodenum were measured through saline-filled open-tip catheter (papers I IV VI VII and IX) In order to obtain sensitive continuous recordings of the activity and the flow resistance of the sphincter of Oddi a perfusion method was developed. The isolated sphincter (papers II, III IV and V) and the sphincter of Oddi *in situ* (papers (I) VII and IX) were perfused at a low constant rate (3 ml/h or less) and the perfusion pressure was recorded continuously These perfusion rates were considerably lower than those used earlier in similar *in vivo* experiments (e.g Hedner and Rorsman 1969 Liedberg and Halabi 1970) The rates were likewise lower than those applied when the resistance to flow through the sphincter of Oddi (isolated or *in situ*) was measured using a drop counter (e g. Benzi Berté Crema and Frigo 1964 Hedner and Rorsman 1969) A low perfusion rate was suggested to be of importance in achieving minimum duodenal influence on the sphincter activity recordings (paper IX) and it seemed to be of special importance

in the study of sympathetic and sympathomimetic effects on the sphincter of Oddi (see discussion in paper IX)

The recordings obtained with the open-tip catheter in the duodenal lumen were considered insufficient for the measurement of intestinal activity close to the sphincter of Oddi. Therefore a new method was developed (paper VII) Pieces of the femoral veins were taken and inserted through the duodenal wall around the sphincter of Oddi. The venous insertions were perfused in a similar manner to the sphincter The perfusion pressure reflected sensitively and selectively the intramural duodenal pressure close to the sphincter of Oddi (papers VII and IX)

Hormonal regulation of biliary motility

During interdigestive periods the continuously secreted liver bile enters the gallbladder The resistance of the sphincter of Oddi is of significant aid to this process (Hallenbeck 1967) It is not definitely established whether any nervous or hormonal mechanism is of importance during this gallbladder filling phase As will be discussed below the results obtained in paper IX suggest that the activity of the adrenergic nervous system promotes the entry of bile into the gallbladder

The mechanism of evacuation of the gallbladder can be explained as being due to hormonal effects. Boyden (1923) showed that the gallbladder emptied in response to food (fat) intake Whitaker (1926) demonstrated that the denervated gallbladder would also evacuate when food was ingested Ivy and Oldberg (1928) made an extract from upper intestinal mucosa which upon i.v injection brought about the contraction and emptying of the gallbladder These authors named the extracted hormone cholecystokinin Sandblom, Voegtlin and Ivy then (1935) made the observation that CCK relaxed the sphincter of Oddi. These dual actions of CCK have been confirmed many times in different species (reviewed by Grossman 1950 Hallenbeck 1967 Jorpes and Mutt 1969) In the present work it was found that CCK consistently relaxed the sphincter of Oddi and contracted the gallbladder (papers I, II V VII VIII and IX) Recently (K.E. Andersson, R. Andersson, Hedner and C.G.A. Persson 1972) it was demonstrated that prostaglandin E_2 (PGE_2) shared with CCK the dual effects on biliary muscle Further investigations are, however required before possible roles of PG.s in biliary dynamics can be discussed

CCK has also been shown to have contractile effects on the intestinal muscle (Dahlgren 1967 Hedner H Persson Rorsman 1967 Jorpes and Mutt 1969 Ramirez and Farrar 1970) Of special interest in biliary dynamics is the effect of CCK on the duodenal tissue that surrounds the sphincter of Oddi. Using the method with perfused venous insertions (paper VII) actions on this part of the duodenum could be selectively recorded. In these studies, CCK was shown to relax the duodenal tissue close to the sphincter of Oddi. This relaxation recorded as decreased intramural pressure in the duodenum, occurred simultaneously with the relaxation of the sphincter of Oddi. A duodenal influence on the sphincter concomitant with the latter effect might then be defined as a facilitation of the sphincter

relaxation Earlier a number of reports had shown a biphasic response to CCK including a contraction of duodenal tissue (reviewed by Dahlgren 1967 Jorpes and Mutt 1969) Species differences may have contributed to these conflicting results (cf Bertaccini and Agosti 1971) but methodological dif ferences may also be of importance If the method does not record selec tively the activity of the part of duodenum that surrounds the sphincter of Oddi, the activity of more distal parts of the intestine may influence the recording Actions of CCK on intestinal tissue distal to the sphincter of Oddi seem well defined as contraction and increased peristaltic activity (Hedner H. Persson and Rorsman 1967 Ramirez and Farrar 1970 Frigo Torsoli Lecchini, Falaschi and Crema 1972) In papers II and VII it was confirmed that CCK can contract intestinal smooth muscle distal to the choledochoduodenal junction

Mechanism of action of cholecystokinin

Recent investigations have suggested a probable mechanism involved in the actions of CCK. CCK seems to act directly on the smooth muscle cells in the biliary tract since its effects are not mediated by the cholinergic or the adrenergic nervous systems. This has been shown both for the gallbladder by Ivy and Oldberg 1928 Hedner 1970 and in paper IX, and for the sphincter of Oddi in the present studies (papers I II and IX)

Many hormones are known to change the tissue levels of cyclic AMP (cAMP) and this nucleotide has been proposed as an intracellular mediator of hormonal effects (Robison Butcher and Sutherland 1971) Amer (1970) found that CCK stimulated phosphodiesterase (PDE) prepared from the gallbladder PDE is the cAMP inactivating enzyme and a stimulation of PDE should result in decreased tissue levels of cAMP Andersson et al. (K.E. Andersson R. Andersson and Hedner 1972) produced direct evidence that CCK decreased the cAMP level in the gallbladder tissue and confirmed the stimulant effect of CCK on the gallbladder PDE. These authors used the C-terminal octapeptide of CCK (C8-CCK) in their study on cholecystokinetic effects on the gallbladder This peptide is known to produce the same mechanical and secretory effects as the extracted hormone (Mutt and Jorpes 1968 Rubin, Engel, Drungis, Dzelzkalns, Grigas, Waugh and Yiacas 1969 Hedner 1970 and paper IX)

C8-CCK was also used in studies of metabolic and mechanical effects on isolated sphincter of Oddi preparations (paper VIII) C8-CCK relaxed the sphincter as did CCK. In contrast to the finding in the gallbladder C8-CCK was seen to increase the tissue concentration of cAMP in the sphincter The increase of the cAMP content preceded the relaxation and was probably brought about by stimulant effects of C8-CCK on adenylcyclase, the enzyme responsible for the formation of cAMP PDE in the sphincter muscle showed increasing activity during contact with C8-CCK.

Other agents known to influence the formation or breakdown of cAMP produced the same mechanical effects in the sphincter of Oddi (papers I II and VIII) as they did in the gallbladder (Amer 1969 paper VI and K.E. Andersson R. Andersson and Hedner 1972) These agents included adenyl cyclase stimulating (β-receptor stimulants and glucagon) PDE-activating (imidazole) and PDE-inhibiting (theophylline and papaverine) drugs (Table 1) According to the proposed mechanism of action of these compounds (for

review see Robison Butcher and Sutherland 1971) a relaxation of biliary smooth muscle is associated with an increased intracellular cAMP concentration while contraction is associated with decreased cAMP content in the tissue Relaxation was accordingly also obtained when cAMP was added to the gallbladder (K.E. Andersson R. Andersson and Hedner 1972) and sphincter of Oddi preparations (paper VIII)

Table 1

	Tension		cAMP	
	GB	SO	GB	SO
ß-receptor stimulants	↓	↓	[↑]	[↑]
Glucagon	↓	↓	[↑]	[↑]
Theophylline	↓	↓	[↑]	[↑]
Papaverine	↓	↓	[↑]	[↑]
Imidazol	↑	↑	[↓]	[↓]
C8-CCK	↑	↓	↓	↑
PGE_2	↑	↓	↓[1]	↑[1]
cAMP	↓	↓		

Table 1
This table summarizes metabolic effects (changes in cAMP content) and mechanical effects (tension changes) of drugs on the gallbladder (GB) and the sphincter of Oddi (SO). The arrows directed upwards represent positive changes: increases and stimulant effects Downward directed arrows represent the opposite Arrows within brackets are postulated effects that have not been determined
1 Unpublished observations by K.E. Andersson, R Andersson P Hedner and C.G.A Persson.

The effects on the cAMP level induced by C8-CCK and the effects produced by cAMP and the various agents known to influence the formation and breakdown of cAMP are consonant with the idea that tissue levels of cAMP and the mechanical activity of biliary smooth muscle can be causally related. It is generally accepted that the level of free myoplasmic calcium ions regulates the contractile activity of a muscle (Sandow 1965 Ebashi and Endo 1968 Sandow 1970) R. Andersson and K. Nilsson (1972) found that cAMP stimulated the activity of a calcium-accumulating microsomal

fraction of intestinal smooth muscle This observation suggests a mechanism by which the intracellular concentration of free calcium ions can be in fluenced (R. Andersson Lundholm, Mohme Lundholm and K. Nilsson 1972) Therefore in the gallbladder and in the sphincter of Oddi the intra cellular content of cAMP may be involved in a regulation of free calcium ions. Changes in the cAMP-level may then be of relevance for the initiation of a mechanical response The importance of a cAMP-dependent Ca^{++} accumulating mechanism for the contractile activity of various smooth muscles has not been settled. For example, results obtained in uterine muscle are not in favour of a general involvement of cAMP in the regulation of contractile changes in smooth muscle (Batra and Daniel 1971 a, Batra and Daniel 1971 b Polacek, Bolan and Daniel 1971 Polacek and Daniel 1971)

Nervous regulation of biliary motility

A Parasympathetic effects

The biliary tract is supplied with parasympathetic nerves through the vagus (Hirt 1934 Rothman 1965) Vagal denervation of the upper abdominal viscera is a common surgical procedure and the role of the vagus in biliary dynamics has received special attention (Pallin and Skoglund 1961 Beneventano Rosen and Schein 1969 Liedberg 1969 Williams and Huang 1969 Amdrup and Griffith 1970 Fagerberg, Grevsten, Johansson and Krause 1970 Schein and Gliedman 1970 Loeweneck 1971) However it has not been established whether the vagus is involved in the normal function of biliary motility nor whether vagotomy is causally related to any biliary disease (Hallenbeck 1967 Bouchier 1970)

Acetylcholine contracts both the gallbladder and the sphincter of Oddi (Hallenbeck 1967 papers II VI and IX) This finding and the results of nerve stimulation suggest that the vagus mainly provides the biliary tract with motor fibres (Ivy 1934 Pallin and Skoglund 1961 Crema Berté, Benzi and Frigo 1964 Pallin and Skoglund 1964) The contraction induced by vagal stimulation cannot by itself evacuate the gallbladder (Ivy 1934) but may increase the contraction of the gallbladder produced by CCK (Pallin and Skoglund 1964) Crema, Berté Benzi and Frigo (1964) report and review constrictor responses of the choledochoduodenal junction during vagal stimulation These observations agree with the finding that acetylcholine contracts the sphincter of Oddi (Crema, Benzi and Berté 1962 Hallenbeck 1967 and papers II and IX) *in situ* and in the isolated preparation.

B. Distribution and function of adrenoceptors in the gallbladder and the sphincter of Oddi

Ahlquist (1948) advanced the concept that adrenergic neurotransmitters and sympathomimetic agents act on special receptors (adrenoceptors) classified by him as α and β-receptors. The differentiation of these receptors was based on the rank order of potency in various organs of 6 sympathomimetic amines. This classification has proved most useful and now highly selective stimulants and blockers of α and β-receptors are known The definition of α and β-receptor effects has formed a basis for a better understanding of possible adrenergic functions in various organs.

In determining the presence of α and β receptors in the gallbladder and the sphincter of Oddi various adrenoceptor agonists and antagonists were

used (papers I—IX) Both the gallbladder and the sphincter of Oddi are shown to contain contraction-mediating α receptors and relaxation mediating β-receptors (papers I II VI and IX) However the gallbladder (isolated and *in situ*) seems to have only a small population of α receptors as the α-receptor mediated contraction in response to noradrenalin and adrenalin was weak and could only be obtained when the β receptors had been blocked A similar small population of excitatory α receptors has earlier been described for bronchial (H Persson and Johnson 1970) and urinary bladder detrusor (Edvardsen and Setekleiv 1968) smooth muscles. In the untreated (no β-blocking agent present) isolated sphincter of Oddi the adrenergic amines (noradrenalin and adrenalin) consistently produced contraction and increased activity (Magee 1946 Crema Benzi and Berte 1962 and paper II) In contrast to the findings of other authors (Benzi, Berté Crema and Frigo 1964) the present work confirmed this excitatory α receptor function in the cat sphincter of Oddi *in situ* (papers I VII and IX) The sphincter of Oddi thereby resembles other sphincters along the gastrointestinal tract (Munro 1951 Gazet and Jarret 1964 Christensen and Daniel 1966 Parks Fishlock Cameron and May 1969 and Kerremans and Penninckx 1970) where excitatory responses to α-receptor stimulation have been seen The major part of the intestine otherwise contains α and β-receptors which both mediate relaxation (Ahlquist and Levy 1959) This synergism between adrenoceptor functions seems to apply also to the duodenal tissue surrounding the sphincter of Oddi (papers II VII and IX) Most intestinal muscle is thus, by the α-receptor mediated relaxation, distinguished from biliary smooth muscle where contraction mediating α receptors were found (papers II and VI)

There is pharmacological evidence (Paton and Vizi 1969 Kosterlitz, Lydon and Watt 1970) supported by morphological findings (Norberg 1964) that an inhibitory α receptor is situated on cholinergic ganglia or axons in the intestine However intestinal smooth muscle seems also to contain inhibitory α receptors (Bowman and Hall 1970) It is possible that adrenoceptors are localized to neuronal structures as well as to smooth muscle cells in the biliary tract. The present results, however did not suggest the presence of inhibitory α receptors in the gallbladder or the sphincter of Oddi (papers II, VI and IX)

In terms of distribution and function of adrenoceptors it may be of interest to compare the extrahepatic biliary tract with the urinary bladder Both organs have a storage function and intermittently expel their contents. In the urinary bladder the detrusor muscle was shown to contain mainly β-receptors which were inhibitory in function noradrenalin and adrenalin relaxed the detrusor (Edvardsen and Setekleiv 1968) This corresponded

well to the findings with gallbladder muscle (paper VI) The urinary bladder base similarly to the sphincter of Oddi responded with contraction to the adrenergic amines. Based on this finding a sphincter function was proposed for the bladder base (Edvardsen and Setekleiv 1968) Various parts of the gallbladder however showed a uniform adrenoceptor pattern (VI) This finding lends no support to a sphincter function in the gallbladder collum (cf Rothman 1965) Such a function of the sphincter of Oddi is then stressed by the similarity to the urinary bladder base concerning the adrenergic response as well as to other sphincters along the gastrointestinal tract (discussed above)

In view of the therapeutic interest in pharmacological agents that relax biliary smooth muscle, the sensitivity of gallbladder and sphincter of Oddi preparations to a selective β receptor stimulating drug terbutaline (Bergman H Persson and Wetterlin 1969) was studied

Lands et al (Lands and Brown 1964 Lands, Groblewski and Brown 1966 Lands, Arnold McAuliff Luduena and Brown 1967 Lands, Luduena and Buzzo 1967) studied the effect of β-receptor stimulating agents on various organs. Based on potency rank order differences (as in Ahlquist s study 1948) between the compounds in different organs, they suggested that different β-receptors exist, β_1 (e.g in the heart) and β_2- (e g in the lung) H Persson and co-workers (H Persson and Johnson 1970 H Persson and Olsson 1970) have substantiated the presence of different β-receptors in investigations with different muscles (heart and lung) from the same animal under identical experimental conditions. Further support for the subdivision of β-receptors is given by recently characterized selective (on lung) β_2-receptor agonists such as terbutaline (H Persson and Johnson 1970) and selective (on heart) β_1 receptor antagonists such as practolol (Levy and Wilkenfield 1969) With terbutaline it has been confirmed that β_2-receptors are also present in the vascular system (H. Persson and Olsson 1970) in skeletal muscle (Olsson 1972) and in the uterus (Olsson and C.G.A Persson 1971)

The potency of terbutaline in relaxing biliary smooth muscle was compared with that of another nonselective β-receptor agonist, isoprenaline Terbutaline was shown to relax the gallbladder and sphincter of Oddi preparations to the same extent as isoprenaline (papers II and VI) Terbutaline thus seemed to be a full β-receptor agonist on biliary muscles (papers II and VI) The potency ratios between terbutaline and isoprenaline obtained in gallbladder and sphincter of Oddi preparations suggested that, compared to isoprenaline terbutaline was more active on biliary smooth muscle (papers II and VI) than on heart muscle (H Persson and Olsson 1970) It was further demonstrated that terbutaline counteracted the spasmogenic effect

of morphine at the choledochoduodenal junction (paper VII) This effect could be reproduced in humans where it was shown that terbutaline decreased the intrabiliary pressure that had been increased by morphine (Ingemarsson Liedberg and C.G.A. Persson 1972) Isolated human gallbladder preparations could also be relaxed by terbutaline (paper VI)

C. Effect of adrenergic nerve stimulation on the gallbladder and on the sphincter of Oddi

The splanchnic nerves are the main source of sympathetic innervation to the upper abdominal viscera in the cat (Hirt 1934) Baumgarten and Lange (1969) using the Falck Hillarp fluorescence technique have shown that adrenergic nerves, probably containing noradrenalin are present in the gallbladder and the sphincter of Oddi of the cat. Results obtained with tyramine noradrenalin and adrenalin on isolated gallbladder strips taken from normal and reserpinized cats suggest that the adrenergic nerves relax the gallbladder (paper VI) Similar studies performed on the isolated sphincter of Oddi show that the sphincter is contracted when the adrenergic transmitter amine is released by tyramine (paper II) These adrenergic effects on biliary smooth muscle can be explained in terms of adrenoceptor population and functions in the gallbladder (papers VI and IX) and the sphincter of Oddi (papers I II and IX) as discussed above

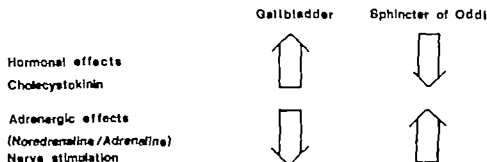
In agreement with the *in vitro* findings it was shown that stimulation of the right great adrenergic splanchnic nerves, as well as i v noradrenalin, mainly activated α-receptors in the sphincter of Oddi (inducing contraction) and β-receptors in the gallbladder (inducing relaxation) (paper IX) The finding that stimulation of the right splanchnic nerves relaxes the gallbladder agrees with the results obtained by Bainbridge and Dale (1905—1906) and Pallin and Skoglund (1964) It seems, however that the gallbladder pressure has to be elevated (e.g. by CCK) before a clear-cut relaxation is regularly obtained by stimulation of the adrenergic nerve supply to the gallbladder (paper IX)

Inconsistent responses to noradrenalin, adrenalin and adrenergic nerve stimulation in the sphincter of Oddi are reported in the literature (for reviews see Ivy 1934 and Hallenbeck 1967) As mentioned under Experimental Approach and discussed in paper IX, it should be of particular importance to have a method which selectively records the resistance of the sphincter of Oddi when sympathetic and sympathomimetic effects are evaluated. This reasoning seemed valid because the contiguous duodenal musculature is relaxed by adrenergic stimuli including α receptor activation (papers II and IX) while judging from *in vitro* and *in situ* experiments, the

sphincter of Oddi is contracted in response to such stimulation (papers I, II and IX) In paper IX it is discussed how the methods employed (e g higher perfusion rates through the sphincter than used in the present work) may have contributed to the difficulties experienced by other authors (Benzi Berté, Crema and Frigo 1964 Crema Berté, Benzi and Frigo 1964) in confirming *in situ* the contraction that is produced by the adrenergic amines in isolated sphincter of Oddi preparations (Magee 1946, Crema, Benzi and Berté 1962 and paper II)

From the studies presented in paper IX it was moreover evident that adrenergic stimuli, in relaxing the gallbladder counteracted the CCK induced contraction. In the sphincter of Oddi the situation was the reverse CCK produced relaxation and adrenergic stimuli contraction These findings showed that the effects of splanchnic adrenergic innervation counteracted hormonal effects on biliary motility (Fig. 2)

Fig 2 Hormonal and adrenergic reciprocal contractile effects on extra-hepatic biliary motility



In summary the dual adrenergic effects on the gallbladder (relaxation) and the sphincter of Oddi (contraction) are produced by the adrenergic amines (noradrenalin and adrenalin) both in isolated preparations and in *in vivo* studies the dual effects are obtained through release by tyramine of the adrenergic transmitter in the isolated organs and also *in situ* when the adrenergic nerve supply to the biliary tract is stimulated. Finally the dual effects are explained by the finding that in the gallbladder the β-adrenoceptors dominate while in the sphincter there is a more even distribution between α and β-receptors It is possible that the adrenergic effects are of significance during the gallbladder filling phase, promoting the entry of bile into the gallbladder

Nature and function of the spontaneous activity of the sphincter of Oddi

It has been observed by many workers that the sphincter of Oddi exhibits spontaneous activity (Lueth 1931 Magee 1946 Bergh and Layne 1940 Crema, Benzi and Berté 1962, Wyatt 1967) In studies II III V and VII various patterns of spontaneous sphincter activity are shown In the *in vitro* experiments both tension changes in the longitudinally mounted sphincter of Oddi and resistance to flow through the sphincter (by constant rate per fusion through a cannula in the distal common bile duct) are recorded. Each change in tension, spontaneous or drug-induced, was accompanied by a cor responding pressure change This parallelism was seen in detail because the constant rate perfusion of the sphincter provided continuous recording of the resistance to flow through the sphincter Longitudinal tension and re sistance to flow seemed to be interrelated because of the parallel recordings and because mechanical stretching of the sphincter preparation, simulating the longitudinal movements of the sphincter did not cause changes in the resistance to flow (paper II) The spontaneous activity of the sphincter of Oddi was shown to be myogenic in origin as it was not inhibited by agents known to block nervous effects (paper II) In this respect the sphincter is comparable in behaviour to many other visceral smooth muscles (Holman 1968) *In situ* the sphincter showed a spontaneous activity that resembled the *in vitro* recordings (papers I and IX) The sphincter activity *in situ* was also affected by pharmacological agents in the same way as the isolated preparation (papers I, II and IX)

Boyden (1957a) in his study on muscle arrangements in the cat choledochoduodenal junction reported that, in the proximal part of the intra mural sphincter of Oddi the pancreatic and bile ducts are separated by a muscle septum, which according to him might selectively influence the bile duct. This report made it also of interest to study the resistance to flow through the pancreatic duct, by the isolated sphincter of Oddi (paper V) Both pancreatic and bile ducts were perfused simultaneously in the longitudinally mounted sphincter preparation (paper V) Perfusion pressures and isometric tension were recorded A close parallelism between the three recordings, tension changes and changes in both perfusion pressures was shown. This concerned both the spontaneously active sphincter and the

sphincter under the influence of drugs (paper V) This finding lends no support to a functional significance of the possibility pointed out by Boyden (1957a) that pancreatic and bile flow may be selectively affected by the sphincter However the results can be linked with the view that the cat sphincter of Oddi is a functional syncytium as discussed by Dewey and Barr (1968) for smooth muscle organs.

Various opinions have been expressed on the role of the sphincter of Oddi in the transport of bile into the duodenum. Watts and co-workers (Watts and Dunphy 1966 Toouli and Watts 1972) suggested that the sphincter acts as a pump activated by CCK. They performed experiments on the dog sphincter of Oddi *in situ* and *in vitro* It may be said in this connection that Boyden in his anatomical investigations found more similarities between cat and human sphincter of Oddi than between dog and human sphincters (Boyden 1957a) Watts and Dunphy (1966) in their *in situ* study have actually illustrated a biphasic response to CCK where the effect with the longest duration is a relaxation of the sphincter In the isolated sphincter of Oddi, Toouli and Watts (1972) obtained no response to CCK in four out of nine preparations. In three, slight contractions were obtained and in one preparation CCK inhibited the spontaneous activity It seems then, that the conception that CCK relaxes the sphincter of Oddi as substantiated by other workers (Sandblom, Voegtlin and Ivy 1935 Magee 1946 Crema, Benzi and Berté 1962, Wyatt 1967 Hallenbeck 1967 Hedner and Rorsman 1969) is better founded than the view presented by Watts and co-workers (Watts and Dunphy 1966 Toouli and Watts 1972)

In the isolated perfused sphincter of Oddi it was consistently shown that increased spontaneous activity or drug induced activity was associated with increased resistance to flow through the sphincter (papers II and V) and CCK consistently inhibited the sphincter activity and decreased flow resistance (papers II and V) The metabolic effects of CCK (paper VIII) support this finding. When the perfusion tube was disconnected from the perfusion apparatus and held so that the perfusion pressure was kept constant the spontaneous activity of the sphincter of Oddi was insufficient to decrease the fluid level in the tube (paper II) Thus it was shown that the sphincter activity has no propulsive function but is associated with increased resistance to flow through the sphincter

Tetrodotoxin (TTX) was found to increase the spontaneous activity of the isolated sphincter of Oddi (paper III) TTX also seemed to potentiate the excitatory effects of acetylcholine and noradrenalin (paper III) TTX has been shown to abolish action potentials by interfering with the sodium permeability in nerve fibres (reviewed by Kao 1966) Smooth muscle re

activity is not affected by this action and thus TTX is recommended as a convenient denervating agent in experiments on isolated smooth muscle organs (Gershon 1967) The excitatory effect of TTX on the sphincter of Oddi seemed difficult to explain by actions on nerve structures (paper III) However Wood (1972) quite recently studied excitatory electrophysiological and mechanical effects of TTX on circular intestinal muscle He produced evidence that the stimulant effect of TTX was not directly on the muscle but involved inhibition of spontaneously active inhibitory neurones. If this was true for the effect of TTX on the sphincter of Oddi, the spontaneously active inhibitory neurones would neither be cholinergic nor adrenergic in nature because no consistent change in the sphincter activity was seen when effective doses of atropine and adrenoceptor blocking agents were added to the isolated sphincter (papers II and III) The contractile effect of TTX might then be regarded as indicating a localization of non-adrenergic inhibitory nerves to the sphincter of Oddi. There is cumulating evidence for the presence of non-adrenergic inhibitory nerves as well as of non-cholinergic excitatory nerves in the gastro-intestinal tract (reviewed by Bortoff 1972) To my knowledge no studies on the presence and function of non-adrenergic and non-cholinergic nerves in the biliary tract have been performed

Mechanism of action of morphine at the choledochoduodenal junction

Morphine and related analgesics have important effects on biliary dyna mics. They increase the intraductal pressure in the biliary tract This effect is due to increased resistance to flow through the choledochoduodenal junction (for reviews, see Hallenbeck 1967 Daniel 1968) It is an unwanted side effect of morphine therapy However the morphine induced spasm has also been used for diagnostic purposes (Sörensen 1964) and it is a com mon procedure in the evaluation of spasmolytic effects on human biliary smooth muscle to use morphine as a spasmogenic agent (Hallenbeck 1967 Kewenter and Kock 1971 Ingemarsson Liedberg and C.G.A Persson 1972) Nevertheless, it has not been shown whether the obstruction of the choledochoduodenal junction is due only to the morphine-increased tone *of intestinal muscle or to pharmacological effects on the sphincter of Oddi* as well (paper IV) Since isolated preparations of both duodenum and sphincter of Oddi are insensitive to morphine no conclusion could be drawn from *in vitro* experiments concerning possible effects of morphine on the sphincter (paper IV)

It seemed worthwile to study *in situ* the effect of morphine and the duo- denal influence on the sphincter more closely (paper VIII) In these experi- ments there was good agreement between intensity and duration of the effect of morphine on the perfusion pressures through the sphincter of Oddi and through the venous insertions used for selective recording of duodenal activity close to the sphincter (paper VII) This finding indicated that the morphine increased passage-pressure through the sphincter of Oddi could be due exclusively to an effect on the intestine

The only evidence suggesting that morphine had an effect on the smooth muscle of the sphincter was obtained in studies on the combined effect of morphine plus noradrenalin The point was to make use of the differential effect of noradrenalin on sphincter (contraction) and duodenal muscle (re laxation) A dose of noradrenalin that neutralized the morphine increased duodenal tone was injected during the action of morphine If under these conditions noradrenalin caused the passage pressure through the sphincter of Oddi to rise higher than it did with noradrenalin alone this would in dicate that morphine besides its effect on the duodenum increased the tone

in the smooth muscle of the sphincter of Oddi As reported in paper VII this kind of evidence was obtained in favour of an excitatory effect of morphine on the sphincter of Oddi.

Some other aspects on the spasmogenic action of morphine were dealt with in paper IV It was shown that an effective block of atropine sensitive cholinoceptors and of α-adrenoceptors was insufficient to prevent the morphine spasm These results confirmed the findings of Crema Benzi Frigo and Berté (1965) However in their study they had not included agonists to show that the block was effective It was further shown that the effect of morphine in doses of only 1 μg/kg or less could be counteracted by atropine (paper IV)

Crema, Benzi, Frigo and Berté (1965) working with the cat, did not record any change in sphincter resistance when they infused intra-arterially morphine doses which were even higher than those effective when given intravenously This finding was surprising since other authors have shown that morphine regularly produces spasm of other parts of the intestine when given intraarterially (Daniel 1966 Burks and Long 1967) In contrast to the report by Crema, Benzi, Frigo and Berté (1965) morphine was shown to consistently increase the resistance to flow through the sphincter of Oddi and increase duodenal tone when given intraarterially in about 1/100 of the dose which produces an effect when given intravenously (paper IV) The dose ratio between the two routes of administration is about what can be expected, taking into account the dilution factor of the circulating blood and assuming no special concentration of the drug in choledochoduodenal tissues. This finding suggests that morphine acts by the same mechanism independent of the route of administration It is concluded that morphine exerts its spasmogenic effect locally in the sphincter region through release of some endogenous contracting agent (paper IV)

General summary

The present work has mainly been concerned with adrenergic, cholecystokinetic and morphine induced effects on the cat gallbladder and choledochoduodenal junction. The results obtained can be summarized as follows.

1/ Two methodological developments are of importance a) *In vitro* and *in situ* perfusion of the sphincter of Oddi at a constant low rate resulting in continuous recording of the sphincter activity with minimum influence by the surrounding doudenum b) constant low rate perfusion of venous insertions through the intestinal wall, providing selective and sensitive tracings of local duodenal activity

2/ By the use of relevant adrenoceptor blocking agents, phenoxybenzamine for the gallbladder and propranolol for the sphincter of Oddi, it is demonstrated that CCK does not produce its mechanical effects on extrahepatic biliary smooth muscles by activation of α or β receptors.

3/ CCK is shown to increase the intracellular level of cAMP The metabolic change precedes the mechanical effect and is suggested to be of importance for initiation of the relaxation of the sphincter by CCK The cAMP level is probably increased by stimulant effects of CCK on adenyl cyclase as the enzyme PDE in the sphincter muscle cells shows increasing activity during contact with CCK.

4/ It is shown that other agents which are known to influence the formation or breakdown of cAMP produce mechanical effects on the sphincter of Oddi in agreement with the conception that relaxation is associated with increased and contraction with decreased intracellular levels of cAMP

5/ It is shown that CCK decreases intramural duodenal pressure close to the sphincter of Oddi.

6/ Evidence is provided that both the gallbladder and the sphincter of Oddi contain relaxation-mediating β-receptors and contraction mediating α receptors. In the gallbladder where various parts of the organ were found to have a uniform adrenoceptor pattern the β receptors dominate while in the sphincter of Oddi there is a more even distribution of α and β-*receptors*

7/ The gallbladder and the sphincter of Oddi are found to be sensitive to a selective β_2-receptor agonist terbutaline which seems to be a full agonist in relaxing these organs.

8/ It is demonstrated that the adrenergic amines have dual effects on the

gallbladder (relaxation) and the sphincter of Oddi (contraction) The dual effects are also obtained by release with tyramine of the adrenergic transmitter in the isolated organs and *in situ* when the adrenergic nerve supply to the biliary tract is stimulated It is possible that the adrenergic effects are of significance during the gallbladder filling phase promoting the entry of bile into the gallbladder

9/ The spontaneous activity of the sphincter of Oddi (*in situ* and *in vitro*) is shown to be myogenic in origin The mechanical changes in the sphincter of Oddi (longitudinal tension changes) spontaneous or drug induced are found to correspond in detail with changes in resistance to flow through the sphincter when perfused both through the bile and pancreatic ducts. It is shown that the sphincter activity has no propulsive function but is associated with increased resistance to flow through the sphincter Tetrodotoxin is found to excite the isolated sphinchter of Oddi preparation.

10/ Morphine is shown to be ineffective on isolated preparations of duodenum and sphincter of Oddi. *In situ* a close parallelism is recorded between intensity and duration of action of morphine on intramural duodenal pressure close to the sphincter of Oddi and effect on resistance to flow through the sphincter By simultaneous administration of noradrenalin and morphine data were obtained in favour of the view that morphine increases the tone of the smooth muscle of the sphincter of Oddi In addition it is shown that morphine is potent when given intraarterially and that effective doses of atropine and phenoxybenzamine do not block the spasmogenic effects of morphine.

Acknowledgement

I wish to thank Docent Henry Persson Head of the Pharmacological Department, AB Draco Lund where this work was carried out. He introduced me to experimental pharmacology and during daily discussions I have been confronted with his wide experience in biological research

I am indebted to the management of AB Draco for the excellent working conditions which it has provided.

References

AHLQUIST R.P.L., A study of the adrenotropic receptors. *Am. J Physiol* 1948, 153 586—600.

AHLQUIST R.P and B. LEVY Adrenergic receptive mechanism of canine ileum. *J Pharmac exp Ther* 1959 127 146—149

AMDRUP B.M and C.A. GRIFFITH The effects of vagotomy upon biliary function in dogs. *J surg Res.* 1970 10. 209—212.

AMER, M.S., Mechanism of action of cholecystokinin. *Clin. Res.* 1969 17 520.

AMER, M.S. and G.R. McKINNEY On the mechanism of action of cholecystokinin (CCK). Effect on phosphod esterase (PDE). *Pharmacologist.* 1970. 12. 291

ANDERSSON K.E., R. ANDERSSON and P HEDNER, Cholecystokinetic effect and concentration of cyclic AMP in gallbladder muscle in vitro. *Acta physiol. scand* 1972. 85 511—516

ANDERSSON K.E., R. ANDERSSON P HEDNER and C.G.A. PERSSON Dual effects on gallbladder and sphincter of Oddi induced by cholecystokinin and prostaglandins. *Acta pharmacol.* (Kbh), 1972. 31 suppl. 1 44

ANDERSSON R., L. LUNDHOLM, E. MOHME LUNDHOLM and K. NILSSON Role of cyclic AMP and Ca + in metabolic and mechanical events in smooth muscle In. *Advance in cyclic nucleotide research.* Vol. I. Ed. P Greengard, R. Paoletti and G.A. Robison, Raven Press, New York 1972

ANDERSSON R. and K. NILSSON Relaxation mediated by adrenergic receptors in intestinal smooth muscle role of cyclic AMP and calcium. *Nature* 1972. 238 119—120.

BAINBRIDGE, F.A. and H.H. DALE, The contractile mechanism of the gallbladder and its extrinsic nervous control. *J Physiol* (London). 1905—1906. 33 138—155

BATRA, S C. and E.E. DANIEL, ATP-dependent Ca uptake by subcellular fractions of uterine smooth muscle. *Comp Biochem. Physiol.* 1971 38 A. 369—385

BATRA, S.C and E.E. DANIEL, Effect of multivalent cations and drugs on Ca uptake by the rat myometrial microsomes. *Comp Biochem Physiol* 1971 38 A. 285—300.

BAUMGARTEN H G and W LANGE, Extrinsic adrenergic innervation of the extrahepatic biliary duct system in guinea pigs, cats and rhesus monkeys. *Z Z Ilforsch* 1969 100. 606—615

BERGMAN J H. PERSSON and K. WETTERLIN Two new groups of selective stimulants of adrenergic β-receptors. *Experientia.* 1969 25 899—901

BENEVENTANO T C R.G ROSEN and C.J SCHEIN The physiological effect of acute vagal section on canine biliary dynamics. *J surg Res.* 1969 9 331—334

BENZI, G F BERTÉ, A. CREMA and G.M. FRIGO Actions of sympathomimetic drugs on the smooth muscle at the junction of the bile duct and duodenum studied in situ. *B J Pharmacol* 1964 23 101—114

BENZI G and G M FRIGO Sfintere collodistico di Lütkens: Metodo di studio e rilievi farmacologici in vitro. *Il Farmaco* 1963 18 625—629

BERGH G.S The sphincter mechanism of the common bile duct in human subjects. *Surgery* 194_ 11 ?99—330.

BERGH G.S. and J.A. LAYNE, A demonstration of the independent construction of the sphincter of the common duct in human subjects. *Am J Physiol* 1940 128 690—694

BERTACCINI G. and A AGOSTI, Action of caerulein in intestinal motility in man. *Gastroenterology* 1971 60 55—63

BORTOFF A., Digestion Motility *A Rev Physiol.* (Ed Comroe J.H A.C. Giece and R.R. Sonnenschein). 19 34 61—_90

BOUCHIER, J.A.D The gut, the bile and gallstones. *Gut* 1970. 11 799—803

BOWMAN W.C. and M T HALL, Inhibition of rabbit intestine mediated by α- and β-adrenoceptors. *B J Pharmacol* 1970 38 399—415

BOYDEN E.A., The gallbladder in the cat, its development, its functional periodicity and its anatomical variation as recorded in twentyfive hundred specimens. *Anat. Rec* 1923 24 388—389
BOYDEN E.A., The choledochoduodenal junction in the cat. *Surgery* 1957 a. 41 773—786.
BOYDEN E.A., The anatomy of the choledochoduodenal junction in man. *Surgery Gynec. Obstet* 1957 b. 104 641—652.
BURKS, T.F. and J.P. LONG, Release of intestinal 5-hydroxytryptamine by morphine and related agents. *J Pharmac Exp Ther* 1967 156. 267—276.
CHRISTENSEN J and E.E. DANIEL, Electric and motor effects of autonomic drugs on longitudinal esophageal smooth muscle. *Am J Physiol* 1966. 11 387—394
CREMA, A., G BENZI and F BERTÉ, The action of some natural substances on the terminal portion of the common bile duct isolated in toto. *Arch Int Pharmacodyn.* 1962. 137 307—317
CREMA, A., G BENZI G.M FRIGO and F BERTÉ, The responses of the terminal bile duct to morphine and morphine-like drugs. *J Pharmac. exp Ther* 1965 149 373—378.
CREMA, A. and F BERTÉ, Action of sympathomimetic drugs on the isolated junction of the bile duct and duodenum. *Br J Pharmacol* 1963 20. 221—229
CREMA, A., F BERTÉ, G. BENZI and G.M. FRIGO The responses of the sphincterial areas of the extrahepatic biliary tract to the stimulation of sympathetic and parasympathetic nerves. *Acta physiol. lat -amer* 1964 14 24—32.
DAHLGREN, S., The effect of the cholecystokinin on duodenal motility *Acta chl scand* 1967 133 403—405
DANIEL, E.E., Further studies of the pharmacology of the pyloric region. Analysis of the effects of intra-arterial histamine, serotonin, phenyldiguanid, morphine and other drugs on the antrum and duodenal bulb. *Can. J Physiol. Pharmacol.* 1966. 44 981—1019
DANIEL, E.E., Pharmacology of the gastrointestinal tract. In. *Handbook of Physiology Alimentary Canal* (Ed. W Heidel Williams and Wilkins co., Baltimore) 1968 4 2267—2324
DANIELS, B.T., F.B Mc GLONE, H. JOB and R.B SAWYER, Changing concepts of common bile duct anatomy and physiology *JAMA* 1961 178 394—397
DEWEY M.M. and L. BARR, Structure of vertebrate intestinal smooth muscle. In. *Handbook of Physiology Alimentary Canal.* (Ed. W Heidel Williams and Wilkins Co., Baltimore). 1968. 4 1629—1654
DOWDY G.S., Jr The biliary tract. *Lea and Febiger* Philadelphia. 1969
DOYLE, J.S. and J.T FARRAR, A sphincteric mechanism in the cystic duct of dogs. *Int. J Med. Sci Seventh Series.* 1969 2. 109—117
EBASHI S. and M. ENDO Calcium ion and muscle contraction. *Progr Biophys Molecular Biol.* 1968. 18. 123—183
EDVARDSEN P and J SETEKLEIV Distribution of adrenergic receptors in the urinary bladder of cats, rabbits and guinea-pigs. *Acta pharmacol (Kbh)* 1968. 26 437—445
EICHHORN E.P Jr and E.A. BOYDEN The choledochoduodenal junction in the dog. A restudy of Oddi's sphincter *Am. J Anat.* 1955 97 431—460.
FAGERBERG S., S. GREVSTEN H. JOHANSSON and U KRAUSE, Vagotomy and gallbladder function. *Gut.* 1970. 11 789—793
FRIGO G.M., A. TORSOLI, S. LECCHINI C.F PALASCHI and A. CREMA, Recent advances in the pharmacology of peristalsis. *Arch Int. Pharmacodyn.* 1972. 196. suppl., 9—24
GAGE, S.H., The ampulla of Vater and the pancreatic ducts in the domestic cat. (Felis Domestica). *Am. Quart Micro J* 1879 1 126. (Quoted by G.S. Bergh 1942)
GAZET J.C. and R.J JARRET The ileocaeco-colic sphincter· studies in vitro in man, monkey cat and dog. *B J Surg* 1964 51 368—370.
GERSHON M.D , Effects of tetrodotoxin on innervated smooth muscle preparations. *B J Pharmacol* 1967 29 259—279
GLISSON F 1654 Anatomia hepatis. Ed: 3 Hague 1681 (Quoted by G.S. Dowdy 1969).

GRANSER, G G HERTTING E. RISSEL and F WEWALKA, Untersuchungen über die Beinflussbarkeit des Sphincter Oddi. *Arch int Pharmacodyn.* 1956. 105 389—402.
GROSSMAN M.I., Gastrointestinal hormones. *Physiol Rev* 1950. 30. 33—90
GOLENHOFEN K., D v LOH and F.K. LYNEN Intracellular recording of electrical activity in smooth muscle of the common bile duct. *Experientia.* 1971 27 650—652.
HALLENBECK, G.A., Biliary and pancreatic intraductal pressures. In. *Handbook of Physiology Alimentary Canal* (Ed. W Heidel, Williams and Wilkins Co. Baltimore). 1967 2. 1007—1026.
HAUGE, C.W and J.B.D MARK, Common bile duct motility and sphincter mechanism. I. Pressure measurements with multiple-lumen catheter in dogs. *Ann. Surg* 1965 162. 1028—1038.
HEDNER, P., Effect of the octapeptide of cholecystokinin on guinea-pig ileum and gallbladder in vitro. *Acta physiol scand.* 1970. 78. 23 —235
HEDNER, P H PERSSON and G RORSMAN Effect of cholecystokinin on small intestine *Acta physiol. scand* 1967 70. 250—254
HEDNER, P and G RORSMAN On the mechanism of action for the effect of cholecystokinin on the choledochoduodenal junction in the cat. *Acta physiol scand* 1969 76. 248—254
HIRT A., Handbuch der vergleichenden Anatomie der Wirbeltiere, Band 2. Hälft 1 L. Bolk, E. Göppert, E. Kallius, W Lubosch. Berlin. 1934 735—776.
HOLMAN M.E., Introduction to electrophysiology of visceral smooth muscle. In. *Handbook of Physiology Alimentary Canal* (Ed. W Heidel Williams and Wilkins Co., Baltimore). 1968. 4 1665—1708.
INGEMARSSON, I., G LIEDBERG and C.G.A. PERSSON Effect of a β-adrenoceptor stimulating compound (terbutaline) on common bile duct pressure in man. *Acta chir scand* 1972. In press.
IVY A.C., The physiology of the gallbladder Physiol. Rev 1934 14. 1—102.
IVY A.C. and E. OLDBERG A hormone mechanism for gallbladder contraction and evacuation. *Am J Physiol* 1928 86 599—613
JORPES, J.E. and V MUTT Clinical aspects of the gastrointestinal hormones secretin and cholecystokinin. *Scand J Gastroent* 1969 4 49—57
KAO C.Y Tetrodotoxin, saxitoxin and their significance in the study of excitatory phenomena. *Pharmacol. Rev* 1966. 18 997—1049
KERREMANS, R. and F PENNINCKX, A study in vivo of adrenergic receptors in the rectum and in the internal anal sphincter of the cat. *Gut* 1970. 11 709—714
KEWENTER, J and N.G KOCH, The effect of some spasmolytic drugs on the choledochoduodenal junction in man. *Scand. J Gastroent* 1971 6. 401—405
KOSTERLITZ, H W RJ LYDON and A.J WATT The effect of adrenaline nor adrenaline and isoprenaline on inhibitory α- and β-adrenoceptors in the longitudinal muscle of the guinea pig ileum. *B J Pharmacol* 1970. 39 398—413
LANDS, A.M A. ARNOLD J P McAULIFF F.P LUDUENA and T G. BROWN Jr Differentiation of receptor systems activated by sympathomimetic amines. *Nature* 1967 214 597—598.
LANDS A.M and T G BROWN Jr A comparison of the cardiac stimulating and bronchodilator actions of selected sympathomimetic amines. *Proc Soc exp Biol* 1964. 116 331—333
LANDS, A.M G.E. GROBLEWSKI and T.G BROWN Jr Comparison of the action of isoproterenol and several related compounds on blood pressure heart and bronchioles. *Arch int Pharmacodyn.* 1966. 161 68—75
LANDS, A M F.P LUDUENA and H.J BUZZO Differentiation of receptors respon- e to isoproterenol. *Life Sci* 1967 6. 2241—2249
LEVY B nd E WILKENFIELD An analysis of selective β-receptor blockade. *Eur J Pharmacol* 1969 5 227—234
LIEDBERG G The effect of vagotomy on gallbladder and duodenal pressures during rest and stimulation with cholecystokinin. *Acta chir scand* 1969 135 695—700.
LIEDBERG G and M HALABI The effect of vagotomy on flow resistance at the choledochoduodenal junction. *Acta chi scand.* 1970. 136. 03—212.

LOEWENECK, H Vagotonie und choledochusinnervation Teil I Die Gef' nerven strassen zum Supraduodenalen Choledochusabschnitt *Der Chirurg* 1971 4 178—180.
LUDWICK, J.R., Observations on the smooth muscle and contractile activity of the common bile duct. *Ann. Surg* 1966 164 1041—1050
LUETH, H.C., Studies on the flow of bile into the duodenum and the existence of a sphincter of Oddi. *Am J Physiol.* 1931 99 237—25_
MAGEE, D.F Some observations on the pharmacology of the sphincter of Oddi. *Quart J Pharm.* 1946. 19 38—43
MUNRO, A.F The effect of adrenaline on the guinea pig intestine. *J Physiol* 1951 112. 84—94
MUTT V and J.E. JORPES, Structure of porcine cholecystokinin pancreozymin I Cleavage with thrombin and with trypsin. *Europ J Biochem* 1968 6. 156—162.
NORBERG K.A., Adrenergic innervation of the intestinal wall studied by fluorescence microscopy *Int. J Neuropharmac* 1964 3 379—382.
ODDI, R., D'une disposition a sphincter speciale de l'ouverture du canal choledoque *Arch. ital. de Biol T* 1887 8 317—3__ (Quoted by G.S. Bergh 194)
OLSSON, T Personal communication.
OLSSON O.A.T and C.G.A. PERSSON Relaxing potency of terbutaline and orciprenaline on rat uterus. *J Pharm. Pharmacol* 1971 23 878—879
PALLIN, B and S. SKOGLUND On the nervous regulation of the biliary system in the cat. *Acta physiol. scand* 1961 51 187—192.
PALLIN, B. and S. SKOGLUND Neural and humoral control of the gallbladder emptying mechanism in the cat. *Acta physiol scand.* 1964 60 358—36_
PARKS, A.G., D.J FISHLOCK, J.D.H. CAMERON and H. MAY Preliminary investigation of the pharmacology of the human internal anal sphincter *Gut* 1969 10. 674—677
PATON, W.D M and E.S. VIZI, The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig ileum longitudinal muscle strip. *B J Pharmacol.* 1969 35 10—28.
PERSSON H. and B. JOHNSON Adrenergic receptors in the guinea-pig trachea and lung. *Acta pharmacol. (Kbh).* 1970. 28. 49—56.
PERSSON, H. and T OLSSON Some pharmacological properties of terbutaline (INN) 1-(3,5-dihydroxyphenyl)-2-(t-butylamino)-ethanol. A new sympathomimetic β-receptor stimulating agent. *Acta med. scand* 1970 suppl. 512. 11—19
POLACEK, I., J BOLAN and E.E. DANIEL, Accumulation of adenosine 3',5'-monophosphate and relaxation in the rat uterus in vitro. *Can. J Physiol Pharmacol.* 1971 49 999—1004
POLACEK, I. and E.E. DANIEL, Effect of α- and β-adrenergic stimulation on the uterine motility and adenosine 3',5'-monophosphate level. *Can J Physiol Pharmacol* 1971 49 988—998.
RAMIREZ, M. and J.T FARRAR, The effect of secretin and cholecystokinin-pancreozymin on intraluminal pressure of the jejunum in the unanaesthetized dog. *Digestive Diseases.* 1970. 15 539—544
ROBISON G.A., R.W BUTCHER and E.W SUTHERLAND Cyclic AMP *Academic Press.* New York and London 1971
ROTHMAN M.M., Anatomy and physiology of the gallbladder and bile ducts. *Gastroenterology* (Ed. H.L. Bockus) 1965 3 567—589
RUBIN B S.L. ENGEL, A.M. DRUNGIS, M DZELZKALUS, E.O GRIGAS H.H. WAUGH and YIACAS, Cholecystokinin-like activities in guinea-pigs and in dogs of the C-terminal octapeptide (SQ 19 844) of cholecystokinin. *J Pharm. Sci.* 1969 58 955—959
SANDBLOM P W.L. VOEGTLIN and A.C. IVY The effect of cholecystokinin on the choledochoduodenal mechanism (Sphincter of Oddi). *Am. J Physiol.* 1935 113 175—180
SANDOW A., Excitation contraction coupling in skeletal muscle. Pharmacol Rev 1965 17 265—320.
SANDOW A., Skeletal muscle. *Ann. Rev Physiol.* 1970. 32. 87—138
SCHEIN C.J and M.L. GLIEDMAN The influence of vagotomy on the normal and diseased gallbladder *Digestion,* 1970. 3 243—250.

SØRENSEN B The morphine transaminase test in biliary dyskinesis. *Ugeskr Laeg* 1964 126. 1393—1395
TOOULI, B. and J Mck. WATTS, Actions of cholecystokinin-pancreozymin, secretin and gastrin on extra-hepatic biliary tract motility in vitro. *Ann Surg* 1972. 175 439—447
WATTS, J Mck and J.E. DUNPHY The role of the common bile duct in biliary dynamics. *Surgery Gynec. Obstet.* 1966. 122. 1207—1218.
VATER, A., In *Haller's disputationum anatomicarium selectarium* Gottinger 1720. 3 259 (Quoted by G.S. Dowdy 1969)
VESALIUS, A., De humani corporis fabrica libri septem. *Basilea* 1543 (Quoted by G.S. Dowdy 1969).
WHITAKER, L.R., The mechanism of the gallbladder *Am. J Physiol.* 1926. 78. 411—436.
WILLIAMS, R.D and T T HUANG The effect of vagotomy on biliary pressure. *Surgery* 1969 66. 353—356.
WOOD J.D Excitation of intestinal muscle by atropine, tetrodotoxin and xylocaine. *Am. J Physiol.* 1972. 222. 118—125
WYATT A.P The relationship of the sphincter of Oddi to the stomach, duodenum and gallbladder *J Physiol. (London)* 1967 193 225—232.

The investigations have been made possible by generous financial support from

First of May Flower Annual Campaign for Children s Health

S mper Nut ition Foundation

Swedish Medical Re earch Council (proj ct No 40X 2203)

Swedish Delegation for Applied Medi al Defence

Smedby Foundation

For ome period during this work I received fellow hip granted by För nade Liv

GENERAL INTRODUCTION

One third to one fourth of man s life is devoted to growth and motor d velop ment Relatively peaking this growth takes place at the same time in the body s different organs and tissues The normal physical activity to which a child is xposed leads to continuous adaptation of thes organs

It is impos ible to foresee the effects urbanisation and a physically less strenuous xistence thanks to technical progres may have on growing individuals A lack of heavy physical stimulation in the teens may lead to impaired development of individual physical capacity and att ndant functions

On th other hand periods of rapid growth i e primarily during pr pube ty and puberty have been regarded as the be t tim s for developing large functional capacity by m ans of phy ical training

In view of th importanc of physical and moto developm nt it is sur prising how littl we know about how a growing individual adapts to exercise and physical training It i my hope that the studi s r ported her may ontribute to greater unde standing in this matter

ISSUES

The studies had two main issu to on id r-

1 the r spons f young individuals to exerci and
2 the ffect of a limited period of physi al training on the re ponse to exercis

In both thes respe ts a comparison will be mad with o responding studies with adults

One aim in the de ign of th tudy was to includ measurement making it possibl to follow the oxygen pathway from th external air to the metaboliz ing mu cl c ll The mu cul tu anaerobic m taboli m was als tudied to some xtent Sub topi s con id ed in thi spect were:

1 the adaptation of ventilation gas xchange and entral i culati n during submaximal and maximal exerci (II IV);
2 the fiber tructure of skeletal mus le enzyme activity and ana robi ne gy supply during xerci (V);
3 the relationship between different body dimension and individual functional apacity (I); and
4 th manner in which th se diff rent function ar aff cted by a period of physi al training (I V)

Th reaction of children to exercise has been th subject of many tudies with respect to both submaximal and maximal exercis conditions The first to study these conditions in detail was Robinson (1938) who m asured inter alia maximal oxyg n uptake (max VO_2) maximal ventilation (max V_E) maximal heart rate (max HR) and blood lactate concentration during maximal exercise by children of different ages Since that time a number of studies have be n performed A tabulation of the result of most of these studi s is provided in Table 1 Of all thes studies Ast and s (Astrand 1952) is one of the mo t valuable ones as it provided a systematic study of th various steps in the oxygen pathway using th methods of measurement available at the time Other Swedish studi s at ubmaximal work loads were made by Bengt son (1956) and Berven (1963)

Of the d tailed studies mad of cardio pulmona y function during exercise by children only one involved the direct det rmination of card ac output (Eriksson Grimby and Saltin 1971) This was also the case for muscle metabolism du ing xercise (Eriksson Karlsson and Saltin 1971) Th two latt r studies disclosed that there are def nite diff rences betw n adults and children

A far a the effect of physical training is concerned two pr nc pal type of studies have be n reported in the literatu e i e cross sectional studi s and longitudinal studi s Large dimensions for the cardio pulmonary vascular syst m w re common features of the form r studies (for ref see Astrand and Rodahl 1970 pp 539 558)

The disadvantage of cross e ctional studies is that the significance of g netic factors cannot be evaluated In this espect longitudinal studies are uperior but the significanc of constitutional fa to s can only be examin d in d tail in studi s of monozygotic twins (Klissou as and Weber 1972)

In 1941 Robinson and Harmon and in 1942 Kn hr Dill and Neufeld pub lished the first studies of the ffect of phys cal training on young men Sev ral such studies have been performed since that time (for ref see Astrand and Rodahl 1970 pp 378 380) An increase in maximal a robic capacity has been noted in stud s made in the past de ade (Rowell 1962 Saltin et al 1968 Ekblom et al 1968 Saltin et al 1969 Kilbom and Astrand 1971) However thi in rease has varied n individual cases f om a per cent or two up to 100 % with a mean value of 15 20 % Th differ c s in incre s s may b due to genet factors to some extent but it has also be n shown that the individual cond tion of ubjects at the start of training is of significance (Saltin et al 1969) In the corre pond ng ma ner ther ha e also been studies of the eff ct of physi al traini g on anaerobic capacity

(H rmansen 1969 Karlsson 1971 and Karlsson t al 1972)

It is l s common with data on childr n Sp ynarova (1966) studied 114 11 yea old boy who w re divided up into thr e group depending on their pre ious activity i boys in track and field clubs boy in bask tball clubs and boys not active in sports Th se boys were tudi d fo thr e yea s with annual det rminations of maximal aerobic and ana robic pow No diff nc could be demonst ated among the thr e groups afte the thr y ars Fo practi al reasons however no accurate ch ck could be made on training frequency and intensity in the two a tive groups Similarly no ch ck could be made to s if the inactive group had be n exposed to any form of physical training

Similar tudies hav c ntly been report d from Isra l (Bar-Or and Zwiren 1972) and the Federal German Republic (Mocellin and Wasmund 1972) with imilar results Howeve training f quency in the two latter tudi was less than in co re ponding studi s of adults This might be one reason why max V_{O_2} failed to in reas

Ekblom (1969a) studied six 11 year old Stockholm boys and aft r 6 and 32 months of t aining he wa abl to demonstrate an incr as in max $\dot{V}_{O_2}$ per height squared of 6 and 25 % r spectively (Table 2) A ont ol group of omparabl boy displayed no such increas

Dani ls and Oldridge (1971) tudied 14 boy during a 22 month pe iod of phy ical training Thi t aining wa supervised by a prof s ional track traine and th boy followed the middle-distanc runners t aining program Howev r no exact h ck on t aining could be mad The boys displ yed no increas in max $\dot{V}_{O_2}$ per kg of body weight but there wa a 5 % incr as in max $\dot{V}_{O_2}$ per height quared (Tabl 2)

Tabl 2 Maximal oxygen uptak befo e (B) aft 6 (A6) afte 22 (A22) and afte 32 month (A32) of phy i al t aining in 11 y old boy (Ekblom 1969) and in 10 15 y ar old boys (Dani ls and Old idg 1971)

Inve tigato		n	Maximal oxyg n uptak		
			$l \times min^{-1}$	$ml \times kg^{-1} \times min^{-1}$	$l \times min^{-1} \times m^{-2}$
Ekblom 1969a	B	6	2 15	53 9	0 965
	A 6	6	2 48	59 4	1 055
	A32	6	3 45	58 1	1 205
Dani ls and Oldridg 1971	B	14	2 33	59 5	1 043
	A22	14	2 84	58 3	1 110

Thus there is some uncertainty about the effect physical training has on young individuals Ekblom s hypothe is (1969b) that rational training during adolescent growth would yield the best result is contradicted by the other studi s which seem to indicate that training in these age groups fails to pro duce any further increase in maximal aerobic power beyond the increase occasioned by growth How ver it is difficult to make any direct comparisons between different studie as training varied consid rably

METHODOLOGICAL CONSIDERATIONS

Subj cts

Eleven year old boys re ident in Stockholm and all attending three parallel classes (5th grade lower secondary school) were asked if they were willing to participate in the study Th boys and their parents were provid d with xt nsive information on the study ts purpos and the possibl risks involv ed Thirteen boys volunteered with the consent of their parents The boys were advised that they were free to b eak off parti ipation whenever they chose but no boy did so On boy who was stricked by a protracted Salmo nella infection making participation in the physical training impossible had to be excluded The other boys r mained healthy throughout th examination period i e Nov mbe 1970 to June 1971 (S ries I) All 12 boys took part in study I 9 in studies II IV and 8 in study V (Table 3) The latter study also included a material comprising 5 boys from the same school and in th same age group (Seri II Table 4)

Tabl 3 Summary of ubject part c pation in the f ve stud (+ parti cipation non part c pat on) Total pota s um not measured in subjects G P P P and Ri S

Subject	Study I	II IV	V
R F	+	+	+
J H	+	+	+
U H	+		
F L	+		
T L	+	+	+
A M	+	+	
B M	+	+	+
G P	(+)	+	+
P P	(+)	+	+
Ri S	(+)		
Ro S	+	+	+
B A	+	+	+

Table 4 Mean valu e with SE and range for some anthropologi al and physiologi al measurements of subjects in Series I and II and in the control group

	Series I	Seri s II	Controls
	n = 12	n = 5	n 16
Age years	11 8 ± 0 2 (11 0 13 0)	11 2 ± 0 1 (10 9 11 5)	11 3 ± 0 1 (10 9 11 7)
Height (H) m	1 510 ± 0 023 (1 375 1 614)	1 457 ± 0 027 (1 385 1 510)	1 438 ± 0 014 (1 360 1 510)
Weight (W) kg	44 5 ± 1 81 (35 0 57 3)	36 7 ± 2 10 (30 6 41 5)	37 6 ± 1 36 (28 0 49 5)
Max HR beats x min 1	196 ± 2 7 (180 210)	204 ± 2 4 (200 213)	200 + 1 6 (187 213)
Max V_E l x min 1	52 6 ± 3 47 (40 7 75 5)	67 2 ± 3 29 (57 6 76 9)	64 6 ± 3 37 (42 1 100 7)
Max V_{O_2} l x min 1	1 86 ± 0 07 (1 52 2 43)	1 93 ± 0 09 (1 72 2 15)	1 84 ± 0 07 (1 35 2 38)
Max $\dot{V}_{O_2}/H^2$ l x min 1 x m 2	0 816 ± 0 031 (0 669 0 979)	0 907 ± 0 014 (0 869 0 943)	0 885 ± 0 019 (0 699 0 991)
Max V_{O_2}/W ml x min 1 x kg 1	42 2 ± 1 78 (31 9 51 6)	52 7 ± 0 97 (50 6 56 2)	49 3 ± 1 23 (39 0 56 2)
Max blood la tate mmol s x l 1	8 0 ± 1 79 (4 5 11 9)	9 4 ± 0 74 (7 2 11 2)	8 6 ± 0 68 (4 6 12 1)

The two group of boys examined did n t epre ent a andom sampling of 11 year old boy In ord to obtain som id as to the ep ntative characte f the two group 16 boy of the ame ag and from th same chool i boys in two parall l clas wer examined The anthropolo gi al and physiological ha a teri ti f both the xperimental group and the cont ol group ar hown in Tabl 4 Thu the 12 boys in S ries I diff ed from the ntrol group omewhat being a f w months old r som what tall r and omewhat h avi On the oth hand max $\dot{V}_{O_2}$ max HR and max lactate c nc ntration value we all mparable Th differenc in

noted was probably du to two boys whose weight exceeded the normal value by + 3 SD when weight was related to h ight (Fig 1) The difference in height was due to three other boys whose height was around the normal valu + 2 SD (Fig 2) This means that Series I was not completely representative with respect to height and weight although functional values appeared to be re presentative of Swedish boys of this age

In order to obtain a better idea of how the two experimental groups of boys were related to other materials comprising boys of the corresponding age a tabulation was made of different domestic and foreign mat rials (Table 1) Thus there w re large differenc s among the diff rent groups studied A median max $\dot{V}_{O_2}$ would be 2 0 $l \times min^{-1}$ 45 $ml \times kg^{-1} \times min^{-1}$ and 0 9 $l \times min^{-1} \times m^{-2}$ However the spread in variation was consid rable from Åstrand s material with Swedish school children (1952) to corresponding values for school children in Philadelphia (Rodahl et al 1961)

G neral procedur

All examinations prior to the period of phy ical t aining in Series I were made in November De mber 1970 and those mad after training were mad from the middl of May to the middle of June 1971 The sam method and techniqu were used before and after training

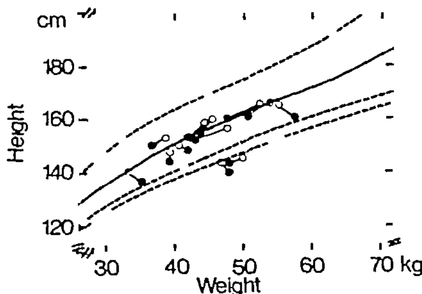


Fig 1 Ind vidual valu s for weight plott d against height befor (filled ymbols) and after 4 months of physical training (unfilled symbol) The solid line indicat s m an weight and broken lines ind ate ± 2 SD and + 3 SD of weight (Broman Dahlberg and Li htenst in 1942)

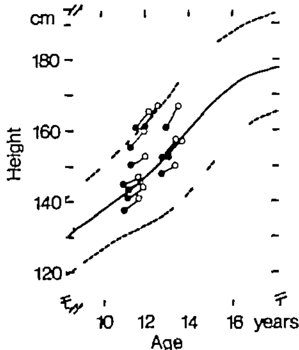


Fig 2 Individual value for height before (filled symbols) and after (unfill d symbols) physical training The solid line indicates mean height and the broken lines indicate ± 2 SD of height for Swedish children (Broman Dahlberg and Lichtenstein 1942)

The levelling-off criterion was used to d fine the max $\dot{V}_{O_2}$ in each pha of the study Thu at least two maximal work loads wer performed on diff nt days both before and after th tudy The values given in Tabl 5 are mean valu s Sinc most of the boys took part in both ardiac output and muscle biopsy studi s during which maximal exerci was performed an extra check was obtained to ensure that a maximal value fo oxyg n uptake r ally had been attained All the studies we performed on a bicycl rgo mete in a sitting po ition Approximately 5 6 % high max V_{O_2} valu are btained fo adults in t admill xerci e (H rmansen and Saltin 1969) Th ame is the as also for children (Saltin and Thorén unpublished sults Table 1) Further support fo the validity of this obs vation is found in the onsistently high r valu found for treadmill tudi s in Table 1

Th boys in Series II were examined at th beginning of Nov mbe and they then immediately tarted th l physical training which lasted until the middle of De embo 1971 at which time new xaminations were mad (A6) Examination wer also mad afte two we k of training (A2)

Physical t aining

The boys in Serie I trained th ee time a week fo more than on hour t a time fo 16 weeks Training always began with a few minute warm up usually in the form of calisth nics followed by running fo about 30 min Training was usually outdoo The running con isted of 3 5 min wo k period

Table 5 Individual values for training frequency and estimated intensity related to the change in maximal oxygen uptake p r unit of height squa ed Training int nsity was graded on a scale of 1 5 with 5 as th highest intensity Numbers in pa entheses indicate total number of training sessions held

Note The April t aining camp is not included in th table

Subj ct	Number of t aining session						Estimated training int nsity	Max $\dot{V}O_2$ A/B corr cted for growth (H^2)
	Jan	F b	March	April	May	Total		
	(5)	(12)	(7)	(7)	(3)	(34)		
R F	5	12	6	4	3	30	2	0 9750
J H	5	12	7	6	1	31	3	1 0838
U H	5	11	7	7	2	32	2	1 0565
F L	5	12	5	5	2	29	2	1 1619
T L	5	10	7	6	0	28	4	1 0963
A M	3	12	7	6	1	29	5	1 4136
B M	5	11	5	7	1	29	5	1 1903
G P	5	11	5	7	1	29	3	1 2420
P P	4	7	7	6	3	27	5	1 1652
Ri S	1	10	7	4	0	22	3	1 2448
Ro S	5	12	6	5	2	30	3	1 0528
B A	5	11	7	6	3	32	4	1 0492
					M an	29 0	3 4	1 14428
					SD	2 7	1 2	0 11182
					SE	0 8	0 7	0 03424

which were nt repeated with a few min rest The speed was close to the highest one they ould maintain for that d stance In addition to this the boys had some print running for bri f period of time Sometimes the cours was run by the boys continuously At the end of the training s ssion the boys played soccer or basketball for 20 30 min When it was heavy rain or snow training was conducted indoors in a gymnasium Th re a p ogram of cali sthenics was performed at a h gh intensity Training was led by an xperi enced gymnastics tea he who had led the training in previous studi s

The boys attended an average of 29 0 (range 22 32) of the 34 training s s ions (Table 5) Their training intensity was evaluated by the trainer on a scale from 0 5 with 5 as th highest intensity The mean value fo intensity was 3 4 See Table 5 for an individual valuation of t aining dilig nce in relation to improvement in max V_{O_2} per h ight squared

In addition to this training a one week t aining amp was also held in th Swedi h mountains which all the boys attended Du ing thi we k training was mor extensiv and mainly consisted of c os country skiing Moreover bandy and tabl tennis were played and there was also downhill kiing and some swimming The boys trained a total of 3-4 hour a day Training inten sity during this week was monitored with a puls tape record r on all th boys Blood amples were also taken for d termination of blood lactate on centration Both these examinations indicated that th intensity achieved during training varied f om 70-80 % of max V_{O_2} to maximal exe tion On the basis of the observations mad during the regular training it was pos ibl to evaluate the intensity during maximal xe tion as being about the same

The 5 boys in Series II trained on a bicycl e gomete thr times a week for six weeks from November to the middl of December 1971 The bicycle wo k consisted of continuous exercis at a work load s lected o that it cor responded to at least 70 % of their max V_{O_2} The mean work load was thus 703 6 kpm x min^{1} (ange 623 798 kpm x min^{1}) Work duration was from 20 50 min (mean 24 2 min) The boy had a mean attendance of 14 4 (ange 11 17) training s s ions out of 18 Howeve one boy wa absent for two weeks be ause of measles Heart rate was checked by a manual count at all training sessions Th boy were in the vicinity of their max HR at each training e sion

Methods

Height and weight were measured mor than one hour aft r th boys g t up in the morning H ight was d termined with the ey ear line ho izontal and with an accurat m asuring instrument (Hart nd n tadiometer) Thr m asur ments of h ight and w ight were mad at ach of the two xaminati n se si ns The ame m asuring in trument we used and th sam pers n mad all the mea ur ments Te ticula volum was measu ed ac ording to Prad r (Prad 1966)

R idual pulmonary volume was establi hed with a clo d h lium sy tem (cf Comro t al 1967) and vital capacity MVV_{40} and $FEV_{1\ 0}$ with a B rnstein spirometer Total Hb and blood volume w re measured with a CO method (Sjöstrand 1948) H art volume wa measured with subject in a ecumbent position (Kj llbe g Rudhe and Sjö trand 1949) Total potas ium was d termined by means of whole body ounting (von Döbeln and Lindell

Table 6 Error of th method for a single determination of oxygen uptake and cardiac output

	Condition	Number of pair d det rminations	Rang		Error of th method %
Cardiac output	Rest	11	4 35	6 83	4 5
$l \times min^{-1}$	Submax exercise	25	5 85	14 94	5 2
	Max exe cise	10	9 07	20 77	4 5
Oxygen uptake	Submax exerci	42	0 76	1 89	1 4
$l \times min^{-1}$	Max exercise	55	1 32	2 86	0 8

1964) Oxygen uptake was d termined at rest and in *xer ise on* a bicycl ergometer using the Douglas bag technique The volume of expiratory air wa measur d in a Tissot spirometer and O_2 and CO_2 concentrations were measured using a modified Haldane technique or a micro-Schollander tech nique with a systematic erro of about one per c nt (Table 6) Heart rate was determined from ECG records Respiratory rate wa counted by auscultatory m ans for two min at rest and fo 30 s c during xercise Blood lactate con c nt ation was established from blood drawn f om a pre warmed fingertip or f om arterial catheters and analysed with a colorimet ic method (St öm s modification (1949) of the Barker Summe s on method (1941)) in tudy I and using an enzymatic method (Scholz et al 1959) fo othe studi s

Arter al blood gas s were d termined from arterial sampl s d awn in a glass syring and analysed w th th a d of a Radiomet r PHM 72 P_{O_2} was establish d with a Clark type mi ro-el ctrod with a polypropylene membrane and P_{CO_2} after micro-equilibration Standard b arbonat values were ob tained from a urve nomogram (Siggaard Anders n t al 1960) Hb con nt ration was e tablished spectrophotometri ally (Holmg en and Pernow 1959)

Th carbon monoxide diffusing capacity of the lungs was established us ng the steady stat method (Filey Mac Intosh and Wright 1954) as modified by Linderholm (Lind rholm 1957) The anatomical right to left shunt was d t r mined through r sp ation of 100 % pure oxyg n (Berggr n 1942)

Cardiac output det rminations were mad using the dye-dilution method with indocyanin green (Cardiogre n®) as the ind cator substanc and a Beckman d nsitom ter as the recording unit Doubl d t rminations we e made for each examination The areas of th curv s obtained w re calculated with planimetry Th systematic er or for cardia output d t rminations w s around 5 % (Table 6) Art rial pressure was d t mined with a pressur

transducer connected to an Elema 81 write-out unit

Mus le biopsies w re taken from th lateral part of the quadriceps f o is muscl The biopsy needl with its cont nts was immediately froz n (within 3 5 s) in liquid nitrogen The wet weight (w w) concent ation of glycogen adenosine triphosphat (ATP) creatin pho phate (CP) glucose glucos 6 phosphate (G 6 P) and lactate were determined according to Karlsson (1971) The mean water content at rest in submaximal and in maximal xercise was 75 7 77 5 and 78 4 % respectively

R sting biopsi s were taken for fiber det rminations and enzyme studie and were p oces ed and analysed according to Gollnick t al (1972) A further description is provided in study V

Statisti s

Conventional statistical methods were used to d termine standard d viation (SD) standard error of the means (SE) m an diff r nc ($\bar{D}$) and t analy s (Sned cor 1967) Paired samples only w re used in the omparison of data obtained before and afte training Furth r statisti al alculatio s were mad in studi s I and III and are reported in detail in the studies

RESULTS AND COMMENTS

Fiber compo ition and enzyme a tivity in skel tal mus le (V)

Results[x)]

Two distinctly diff rent types of mus le fibe can be found in both adult (Gollni k t al 1972) and children Forty five % of th fibe ar heavily stained in myo in ATP as tain The r maining 55 % ar w akly stained Acc rding to Barnard Edge ton and P ter (1970) thi heavy and weak staining is a sociated with slow and fa t cont actil ha a teristics whi h is why the two types of fibe can be d ignated as low twitch (ST) and fa t twitch (FT) fibe s T aining fail d to r sult in any definit chang in the r lative distribution of the two fibe type (Tabl 7) Howev r the oxid tive potential f both fiber type incr a ed a valuated on the basi f DPNH diapho as taining The low twitch fibe w r mo oxidative than the fast twitch fibe *s both befo e and afte training in very individual subj ct*

SDH activity t st was 5 4 µmol x (g x min) [1] prio to t aining A is by 7 % and 30 % was noted afte two and ix w ks f training r spectively (T bl 7) PFK tivity amounted to 8 4 µmol x (g x min) [1] prio to

x) All valu s stated ar mean values

Table 7 Mean values with ± SE for succinate dehydrogenase (SDH) phosphofructokinase (PFK) and muscle fiber population (ST = slow-twitch fibers) in 11 year old boys befo e (B) after 2 (A2) and after 6 (A6) weeks of physical training Valu s from 12 6 and 15 5 year old boys (Eriksson Gollnick and Saltin un published results) from untrained and trained adult men and from top athletes (Gollnick et al 1972) are also included

Material	n	Ag years	SDH	PFK	ST %	$\dot{V}_{O_2}$ max/H^2
			µmoles x (g x min) 1			l x min 1 x m 2
Present group (B)	5	11 2	5 4 ± 0 4	8 4 ± 2 6		0 909
Present group (A2)	5	11 2	5 8 + 0 6	12 5 ± 1 1	54 8 ± 3 4	0 932
Present group (A6)	5	11 3	7 0 ± 0 4	15 4 ± 1 6	48 9 ± 3 4	0 960
Normal boys	9	12 6	4 7 ± 0 5		65 0 ± 4 4	0 977
Normal boys	7	15 5	5 8 ± 0 5		55 1 ± 7 1	1 091
Untrained men	12	24 30	3 6 ± 0 4	25 3 ± 2 1	36 1 ± 5 0	1 024
Untrained men	14	31 52	4 4 ± 0 3	25 2 ± 1 9	43 9 ± 4 8	0 988
Trained men	12	17 30	6 0 ± 0 3	19 9 ± 3 6	52 3 ± 6 8	1 225
Cyclists	4	18 23	11 0 ± 1 0	23 9 + 1 4	61 4 ± 5 9	1 529
Canoeists	4	25 27	5 8 ± 0 9	22 2 ± 4 7	61 4 ± 6 2	1 283
Swimmers	5	18 23	7 6 ± 0 5	22 7 + 0 4	57 7 ± 9 3	1 695
Runners	8	19 23	6 4 ± 0 5	20 1 + 2 5	58 9 ± 3 7	1 553

training After two and six w eks of training increase of 49 % and 83 % re pectiv ly were noted (Table 7) while the RNA content remained unchanged the whol time

Max V_{O_2} fo subj cts in this part of the study (Series II) was 1 93 l x min [1] before training and increased by 2 % and 6 % after two and six weeks of train ing r spectiv ly; h ight and w ight remained unchanged

Comments

Th studied group onsisted of only fiv boys The results must ther fore be interpreted with caution and no gene al conclusions can be drawn The boys displayed a wide spr ad in muscle fiber composition which how v r is in

Study			Maximal oxygen uptake l x min 1 with	without
II	IV	B	1 74	1 85
		A	2 10	2 21
	V	B	1 70	1 87
	"	A	2 09	2 15

Table 8 Maximal oxygen uptake in studies II IV with and without indwelling catheters and with and without mu cle biopsies in tudy V before (B) and after (A) phys cal training

accordanc with conditions in adults (Gollnick et al 1972) However the mean value for ST fibers was somewhat high for thes boys than for sed n tary adults Th same circumstance al o p oved to apply to 12 6 and 15 5 year old boys (Erikss n Gollnick and Saltin unpublished results Tabl 7) The spread in SDH activity was considerably l ss than in muscl fiber distri bution and the mean value was high r than for adults (Tabl 7) It is impor tant to note that PFK activity was trikingly low in comparison with adults (Table 7) From having been about 40 % of th adult value it ro e to 70 75 % of the adult valu afte training Since PFK is regarded as the rate limiting enzym in glycolysis (Danforth and Lyon 1964) thi may be an explanation of the childr n low anaerobic capacity (A trand 1952 Eriks on Karlsson and Saltin 1971)

Oxygen uptake du ing submaximal work (II IV nd V)

Th boys di play d an inc ea e in oxygen uptake which was linear to isi g work load However th absolut valu s fo oxygen uptake at the different wo k load wer 0 1 0 2 1 1 s than the valu stated fo adults (Astrand 1960) Thi can be explained in part by the fact that boys have a lower r sting oxyg n uptake and by the rgometer used However the calculated ffici ncy was 23 26 % This pplied to circumstances befo and afte training and ther was no differenc in thi r spect betwe n valu s befo e and afte training

Maximal aerobi power (I V)

The 12 boys in Series I had a mean max V_{O_2} of 1 86 l x min 1 and th same value was noted fo the 9 boy in studi II IV and the 8 boys in tudy V (Tabl 8) In the more complicated parts of the tudies with indwelling cathe ters and when mu cl biopsies were tak valu s 6 % and 8 % lower respe tively were obtained

After the 16 weeks of phy ical t aining a 19 % higher valu wa obtained

for max $\dot{V}_{O_2}$ and e sentially the same increase was found for the 9 subjects in studies II IV i e 19 % and for the 8 boys in study V i e 15 % Even after training a lower value for max $\dot{V}_{O_2}$ was obtained in the more compli cated parts of the studies i e a value 5 % lower

Comments

If a trial is complicated by extensive measurements it becomes difficult to attain a truly maximal value for oxygen uptake The explanation for this is probably to be found in the experimental conditions themselves Subjects are more restricted by a complicated experiment The 5 % difference found both before and after training in both the cardio pulmonary and muscle biopsy experiments is in good agreement with orresponding studi s of adults (Saltin et al 1968 Ekblom et al 1968 Bjure G imby and Nilsson 1971 Kilbom and Åstrand 1971) Th values present d in studies II V may there for be regarded as being very clos to maximal values

It might also be useful to point out that the increase in max V_{O_2} remains the same irr spective of wh the cathete s w re used or not as long as comparisons were mad with corresponding valu before training

Th t aining effect i e an approximately 20 % increase in max V_{O_2} was somewhat greater than the value found in most studies of adults However correction must be made for the eff ct of growth since the boys were grow ing in the period between the two examination se sions Thus the increase in max V_{O_2} becomes 14 % if max V_{O_2} is expre sed per height squa ed and 16 % if it is exp es ed per kg of body weight These valu s a in good agree m nt with the values found in adults In Ekblom s study (1969 a) of 11 year old boys an increase in max $\dot{V}_{O_2}$ of 6 % was found expressed per h ight squared and 10 % xpr ssed pe kg of body weight afte 6 months of tra n ing However Ekblom s boys had higher initial max $\dot{V}_{O_2}$ valu s whi h may part ally explain th lesse inc ase aft r 6 months of training

Re piratory and ci culatory adaptation to exercise (II IV)

At rest th studied boys had a V_E of 7 1 1 x min ^{1} In exerci e there was a linear increa e at ubmaximal wo k loads However V_E increased more than $\dot{V}_{O_2}$ in maximal work whereby th ventilatory coefficient $V_E/\dot{V}_{O_2}$ incr ased The same ircumstance applied after train ng and was more pro nounc d Thu V_E/V_{O_2} increased by 22 % and max V_E by 49 % In maximal exercise tidal volum V_T was 40 % of vital capacity rising to 52 % aft r training i e V_T/VC nc as d by 30 % (Tabl 9)

Alveolar ventil tion inc ased from 4 6 1 x min ^{1} at st to 48 9 1 x min ^{1} in maximal xercise Similar values w re obtained aft r training exc pt in maximal exercise when alveola ventilation amounted to 59 4 1 x min ^{1} Thus

Table 9 M an val fo some d ta nd tio fo ventilation and int apulmon ry ga x hang at t and du ing xercise Pulmonary diff ing pa ity d t mined at high submaximal load (639 kpm x min 1) B indi ate before phys al training and A aft physical traini g Le els of ignificance betwe n pr and post training valu s are indicated (xxx $p < 0$ 001 xx $p < 0$ 01 x $p < 0$ 05 n s $p > 0$ 05)

Variable	At r t			500 kpm x min 1			Maximal exercis 856 kpm x min 1 B 939 kpm x min 1 A			639 kpm x min 1		
	B		A	B		A	B		A	B		A
V_T/VC %	14 1	n s	14 7	29 0	xx	37 1	40 1	xx	52 4	27 9	xx	41 4
$\dot{V}_E/\dot{V}_{O_2}$	30 1	n	31 1	21 9	xx	27 5	28 9	xx	35 1	23 8	xx	28 3
$\dot{V}_A/\dot{V}_{O_2}$	19 2	n s	17 8	19 2	n s	21 7	28 1	n s	28 4	19 3	x	23 0
$P_{A_{O_2}}/P_{a_{O_2}}$ mm Hg	6	n s	6	6	xx	12	22	n s	23	20	n s	18
$D_{L_{CO}}$ ml x mm Hg 1 x min 1										23 7	n s	24 7
$D_{L_{CO}}/FRC$ ml x min 1 x mm Hg 1 x l 1										20 6	n s	18 2
1 $\dot{Q}s/\dot{Q}$ %	4 4	n s	2 7									

for max $\dot{V}_{O_2}$ and essentially the sam increas was found for the 9 subjects in studies II IV i e 19 % and for the 8 boys in study V i e 15 % Ev n after training a lower value for max $\dot{V}_{O_2}$ was obtained in the more compli cated parts of the studi s i e a value 5 % lower

Comments

If a trial is complicated by extensive m asurements it becomes difficult to attain a truly maximal value for oxygen uptake The xplanation for this is probably to be found in the experimental conditions th mselves Subjects are more restricted by a complicated experiment The 5 % difference found both before and after training in both th cardio pulmonary and muscle biopsy experiments is in good agreement with corr ponding studi s of adults (Saltin et al 1968 Ekblom et al 1968 Bjure G imby and Nilsson 1971 Kilbom and Aatrand 1971) The values presented in studies II V may there for be regarded as being very close to maximal valu s

It might also be useful to point out that th incr as in max $\dot{V}_{O_2}$ r mains the same irrespective of wh ther catheters were used or not as long as *comparisons were mad with corresponding value before training*

The training ffect i e an approximately 20 % increas in max V_{O_2} was somewhat g ater than the value found in most tudies of adults However correction must be made fo the effect of growth since the boys were grow ing in the period between the two examination s s ion Thu the increase in max V_{O_2} becom s 14 % if max V_{O_2} i exp es ed per height squa ed and 16 % if it is expres ed per kg of body weight These valu s are in good agr e ment with th values found in adults In Ekblom s study (1969 a) of 11 year old boys an increase in max $\dot{V}_{O_2}$ of 6 % was found exp essed per h ight squa ed and 10 % expressed per kg of body weight after 6 months of train ing Howev Ekblom boys had higher in tial max V_{O_2} valu s which may partially xplain the lesser increase after 6 months of training

R pi atory and circulatory adaptat on to x rcise (II IV)

At r st the studied boys had a V_E of 7 1 l x min [1] In exercise there was a linear increase at submaximal work loads However $\dot{V}_E$ inc eased more than V_{O_2} in maximal work whereby the ventilatory oefficient V_E/V_{O_2} increased The same circumstance applied after training and was more pro nounc d Thus V_E/V_{O_2} in reas d by 22 % and max V_E by 49 % In maximal exercise tidal volum V_T was 40 % of vital capacity rising to 52 % after training i e V_T/VC in a ed by 30 % (Tabl 9)

Alv olar ventilation n eas d from 4 6 l x min [1] at r st to 48 9 l x min [1] in maximal exe cis S mil r value w re obtained afte training xc pt in maximal exercis when alveola ventilation amount d to 59 4 l x min [1] Thu

and sexes Valu s in parenthes s denote derived data obtained from measure mal heart rates known)

maximal exercise

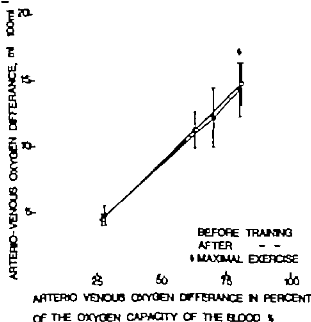
Art. venous oxygen diff ml x 100 ml^{-1}	Transp arterial oxygen l x min^{-1}	AV-diff in % of oxygen bind ing capatity of th blood[x)]	Heart volume ml	Ha moglobin concentration g x 100 ml^{-1}	H ight cm	W ight kg
14 2	2 2	86	500	12 3	150	45
14 7	2 6	88	550	12 4	154	46
14 5	3 2	83	620	13 0	164	51
14 4	4 0	73	720	14 8	178	73
14 3	3 2	87	640	12 2	169	63
14 4	3 7	76	790	14 2	176	76
(13 4)	(2 6)	(76)	800	13 2	175	73
13 7		77	620	13 3	163	60

[x)] Cal ulated from the h moglobin conc ntration at o t

Hb nc nt ation in arterial blood was 12 3 g% and ro to 13 3 g% involving an increase in the blood s oxygen binding capa ity from 16 5 to 17 9 ml x 100 ml^{-1} Similar value we obtained afte training

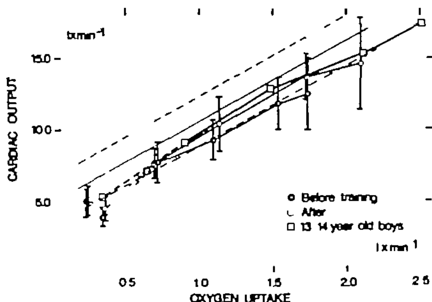
At rest the boys utilized appromimately 30 % of th availabl oxygen binding apacity of the blood in contrast to 80 % during maximal xerci (Fig 3) A d gree of utilization that large h only be n reported fo wom n (A trand t al 1964) who Hb concent ations are omparabl to tho of the studied boys On the the hand adult men utili 70 75 % f the blood s oxygen binding apacity during maximal xerci (Table 10)

The anatomi al right to left shunt in the lung as d t rmined at t wa 4 4 % before training A lowe value i 2 7 % was obtained afte training (Tabl 9)



F g 3 Mean values with ± 1 SD for arterio venous oxyg n difference plotted against arterio venous oxygen differ n e as a per cent of th oxy gen binding capacity of th blood before (filled ymbols) and after 4 months of physical training (unf lled symbols)

Cardiac output at rest was 5 1 x min 1 rising to 10 1 x min 1 at an oxygen uptake of 1 1 1 x min 1 During maximal exercis ard ac output amounted to 12 5 1 x min 1 before training as oppo ed to 14 6 1 x min 1 aft r training (Fig 4) At rest and in submaximal work th was no d fference in ardiac



F g 4 M an values with 1 SD for cardiac output at rest (supine and sitt ng position) and duri g work clud g maximal xercise n 11 13 year old boys before and after 4 months of phy al training Empty squar s denote corresponding mean valu s fo 13 14 yea old boys (Eriksson Grimby and Saltin 1971) Continuous line and lin s with short da h s denote regression line of ardiac output with ± 1 SD for young adult men (Ekblom t al 1968)

output before or after training Thus the arterially transported oxygen during maximal *exercise increased from 2 16 to 2 57 l x min 1 (p < 0 01)* as compared to an increase of 0 36 l x min 1 for max $\dot{V}O_2$

The arterio venous oxygen diff rence increased from 4 5 ml x 100 ml 1 at rest with an increasing work load to more than 14 ml x 100 ml 1 during maximal exercise Values were unchanged after training both at rest and in exercis Thus the maximal a terio venous oxygen difference failed to con ribute to the increase in max $\dot{V}O_2$ The entire increas in max $\dot{V}O_2$ was herefor d e to the increase in maximal oxygen t ansport

H art rate displayed an increas linear to oxyg n uptake in the transition from rest to exercise (Fig 5) A similar increas was noted after training but heart rate was consistently 10 15 beats lower Howeve heart ate during

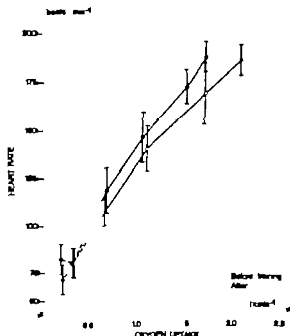


Fig 5 M an valu with ± 1 SD f the heart rate of 11 13 y a old boy at re t (supine and itting po ition) before and aft r 4 mo the f physical training and during xer is in luding maximal xe i e

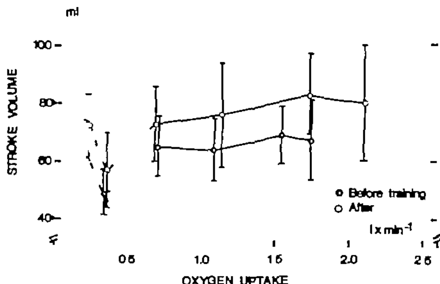


Fig 6 M an values with $^{+}$ 1 SD for the stroke volume of 11 13 year old boys at rest (supine and sitting position) and during exercise including maximal exercise before and after 4 months of physical training

maximal xercise was only 2 beats x min ^{1} lower The heart s stroke volume was 62 ml at rest prior to training and increased to 66 ml in exercise There was no decrea e in stroke volume ven in maximal exercis (Fig 6) Stroke volume was greater after exercise both at r st and in xe cis i e 72 and 77 ml respectively

Comments

Total ventilation in adults increases as work load incr as s (Astrand 1952 Grimby 1969 Bju Grimby and Nilsson 1971) Thus $\dot{V}_E/\dot{V}_{O_2}$ is r latively constant at around 25 during exercise However V_E increases more than $\dot{V}_{O_2}$ as the work load approache a maximal level (Table 11) V_E in thes 11 yea old boys followed a course similar to that of adult men but $\dot{V}_E/\dot{V}_{O_2}$ was a tually lower in both submaximal and maximal exercise (Table 11) In adults the highest V_E s noted at th highest maximal work load (actually at a supermaximal level) The low $V_E/\dot{V}_{O_2}$ in children engaged in maximal exercise may be du to thei lower ana robic capacity and to the attendant r latively lower maximal work load they are capable of performing i e a less pronounced supermaximal work load

$V_A/\dot{V}_{O_2}$ is also somewhat lower in submaximal exercis by thes 11 year old boys as compared to adults but increas s to a comparable level in maxi mal exercis (Table 11) $P_{A_{O_2}}$ $P_{a_{O_2}}$ was lower both at r st and in submaxi

Table 11 T tal ventil tion quival nt ($\dot{V}_E/\dot{V}_{O_2}$) alveol ve til tio quival nt ($\dot{V}_A/\dot{V}_{O_2}$) nd the lveolo a te i l oxyg n tension diff renc (P_{AO_2} P_{aO_2}) in mm Hg in 11 13 y s old boy at e t du ing submaximal and during maximal exercis befo e (B) and aft (A) physical t aining Comparable valu s from ome tudi of adult men are also included in the tabl

Mate ial		At r st			Submaximal xercise			Maximal ex rcise		
		$\dot{V}_E/\dot{V}_{O_2}$	$\dot{V}_A/\dot{V}_{O_2}$	P_{AO_2} P_{aO_2}	$\dot{V}_E/\dot{V}_{O_2}$	$\dot{V}_A/\dot{V}_{O_2}$	P_{AO_2} P_{aO_2}	$\dot{V}_E/\dot{V}_{O_2}$	$\dot{V}_A/\dot{V}_{O_2}$	P_{AO_2} P_{aO_2}
P es nt group	B	30 1	19 2	6	21 9	19 2	6	28 9	28 1	22
	A	31 1	17 8	6	27 5	21 7	12	35 1	28 4	23
11 year old boys (Ekblom 1969 a)	B							31 6		
	A6							32 4		
	A32							32 2		
13 14 year old boys (Erik s n G imby and Saltin 1971)		(49 4)		8	29 9		14	35 0		24
Young adult men (Saltin t al 1968)	B	35 0		14	24 9		20	39 0		31
	A	37 7		9	25 9		14	40 0		25
Young adult men (Ekbl m et al. 1968)	B	37 7			28 0			36 6		
	A	51 9			27 4			36 9		
Middl aged men (H rtl y t al 1969)	B	28 8	24 6	15	25 8	20 3	18	37 2	22 8	26
	A	28 5	21 9	14	25 2	22 5	27	35 8	25 4	28
Middle ged men Bju e Grimby and ils on 1971)		35 7	24 6	21	28 5	26 0	20	38 7	30 0	25

mal exercise but increased to an adult level in maximal exercise (Table 11) On the other hand S_{aO_2} and P_{aO_2} displayed valu s comparable to those of adults as was also th case for D_{LCO} during exercise (Table 9)

The discrepancies displayed by these boys with respect to adaptation of ventilation and ga exchange were less pronounced after training Thus $\dot{V}_E/\dot{V}_{O_2}$ $\dot{V}_A/\dot{V}_{O_2}$ and V_T/VC were then comparable to adult values (Table 11) In this respect Åstrand (1952) wa unable to demonstrate any differenc bet ween children and adults It is impossible to say why these 11 year old boys displayed ventilatory and gas exchange discrepancies One conceivabl ex planation is that the boys in Åstrand s study were relatively well trained i e they had a rather high max $\dot{V}_{O_2}$ in comparison to the pres nt group of 11 year old boys prior to training a diff rence which became l ss apparent after training At the same time as the difference in max $\dot{V}_{O_2}$ declined differences in the adaptation of respiration and gas exchang also disappeared

Cardiac output in submaximal work was 1 2 l less than for young men at the same oxygen uptake (Ekblom et al 1968) The explanation of this is pro bably to be found in a mor efficient distribution of cardiac output to the work ing muscle The arterio venous oxygen difference is then accordingly larger In maximal exercise however the $(a\ \bar{v})\ O_2$ was comparable to adult valu s The max $(a\ v)\ O_2$ difference was also no higher because of the boys' lower Hb concentration and accordingly lower oxyg n binding capacity Since the period of physical training failed to produce any increase in max $(a\ \bar{v})\ O_2$ difference the entire increase in max $\dot{V}_{O_2}$ must have been due to th increase in maximal cardiac output

As is also the case with adults stroke volume was onstant during exer cise and th only increase found was the increas at the t an ition from rest n a sitting pos tion to exercise A slightly larger stroke volume i e 5 ml was found during exercis compared with recumbent re t

Since stroke volume is more or less constant during exercise h art ate b comes responsible for any increase in cardiac output Since cardiac output was somewhat less at the same oxygen uptake fo these boys as compared to adults a i cumstance which also applied to 13 14 year old boys (Eriksson Grimby and Saltin 1971) the calculation of maximal work capacity from work load and h art rate in submaximal exercise must produce inaccurate results *even if corr ction is mad for the children s higher max HR The same* finding has be n report d previously (Hermansen and Os ld 1971)

Heart rate in maximal exercise was more or less unchanged aft r training The change in cardiac output not d aft r training was thus caused by a change in the stroke volum of the h art This stroke volume was cl arly lower than the value for adults but if conside ation is paid to the diff r nce in body size this difference disappears (Table 12) The boys actually dis

Table 12 Maximal stroke volume in ml and maximal stroke volume per height cubed in ml x m ^{3} for 11 13 year old boys before (B) and after (A) physical training Corresponding values from som other studies are also included in the table

Material	Investigators		Stroke volume ml	Stroke volume per H^3 ml x m ^{3}
11 13 yea old boys	Pres nt group	B	67	19 1
		A	80	21 9
13 14 year old boys	Eriksson Grimby and Saltin 1971		87	19 7
Young adult men well trained	Ast and et al 1964		134	23 0
Young adult men	Saltin et al 1968	B	104	16 7
		A	120	20 6
Young adult men	Ekblom et al 1968	B	119	19 1
		A	134	22 6
Young adult men athletes	Ekblom and H rmansen 1968		189	30 8
Middl g d men	Hartl y t al 1969	B	105	18 9
		A	120	22 0
Middle aged men	Bjur Grimby and Nilsson 1971		101	18 2
Middl aged men athlete	Grimby Nil on and Saltin 1966		158	30 5

played a gr ater stroke volume pe height cubed after training than ed ntary adults In thi pect th t aining ff t must be regarded as better in the boy than in adult (Tabl 12)

Dim nsion of the oxyg n transporting and metabolising o gan (I)

Static and dynami pulmonary volume blood volume total Hb h art volum and total pota ium we mea u ed bef and afte training at an int rval of 0 6 year (Table 13) During thi period all th boy had inc as d in height by an ave g of 3 5 cm ($p < 0\ 001$) whil 5 out of 12 of th boys had gone d wn in w ght which i why the weight incre only amounted to 1 1 kg ($p > 0\ 05$) Of th pulmonary volume th re wa nly a 7 % in in TLC and a 4 % in re in VC ($p < 0\ 01$) Total Hb had incr d by 9 % ($p < 0\ 01$) blood volum by 12 % ($p < 0\ 01$) and heart volume by 10 % ($p < 0\ 001$) T tal

Table 13 Mean values with ± SE for dimensions of the oxygen transport and metabolizing organs before (B) and after (A) 16 weeks of physical training Th re were 12 subjects unless otherwise indicated Levels of significance for differ nces betwe n values obtained after and befo e training are indicated (xxx = p < 0 001 xx p < 0 01 x = p < 0 05 n s = p > 0 05)

	M an ± SE		Significance difference
	B	A	A B
Age years	11 8 + 0 2	12 4 + 0 2	
Height m	1 510 + 0 023	1 545 ± 0 026	xxx
Weight kg	44 5 ± 1 81	45 6 ± 1 94	n s
Total lung capacity l BTPS	3 59 + 0 12	3 84 ± 0 16	xx
Vital capacity l BTPS	3 01 + 0 13	3 14 ± 0 13	xx
Functional esidual capacity l BTPS	1 32 ± 0 07	1 49 + 0 08	n
R sidual volume l BTPS	0 64 ± 0 04	0 71 ± 0 03	n s
Forced exp volume 1 s c l BTPS	2 46 + 0 12	2 56 ± 0 14	n s
Max. vol volume (MVV_{40}) $l \times min^{-1}$ BTPS	77 1 ± 2 5	75 0 ± 3 7	n s
Total Hb g	389 ± 10 7	428 ± 18 7	xx
Blood volume l	2 92 ± 0 09	3 28 ± 0 16	xx
Total potassium g	83 81 ± 2 77	95 27 ± 3 61	xxx
H art volume ml	496 ± 28 7	544 + 33 8	xxx
Max $\dot{V}_E$ $l \times min^{-1}$ BTPS	52 9 ± 3 5	80 4 + 5 2	xxx
Max V_{O_2} $l \times min^{-1}$ STPD	1 86 + 0 07	2 21 ± 0 09	xxx
Max h a t rate beats $\times min^{-1}$	198 3 + 2 7	194 3 ± 2 8	x
Max blood lactate mmoles $\times l^{-1}$	8 03 + 0 52	9 52 ± 0 73	xx
RQ	1 02 + 0 01	1 08 + 0 02	xx

Table 14 Mean values for ratios between variables after (A) and before (B) physical training corrected for body size Levels of significance are indicated (xxx $p < 0\ 001$ xx $p < 0\ 01$ x = $p < 0\ 05$ n s = $p > 0\ 05$)

Variables	A/B n = 9		A/B n 12	
	Mean	Significance	Mean	Significance
Weight/H^3	0 945	xx	0 960	xx
Total K/H^3	1 061	xx		
Total Hb/H^3	1 018	n s	1 027	n s
Blood volume/H^3	1 051	n	1 053	n s
Heart volume/H^3	1 012	n s	1 028	n s
Total lung capacity/H^3	1 005	n s	0 994	n s
Vital capacity/H^3	0 966	n s	0 978	n s
Functional residual capacity/H^3	1 100	x	1 058	n s
Residual volume/H^3	1 070	n	1 040	n s
$FEV_{1\ 0}/H^3$	1 008	n s	0 975	n s
MVV_{40}/H^2	0 942	x	0 930	x
Max $\dot{V}_{O_2}/H^2$	1 117	x	1 142	xx

pot ssium incre sed by 14 % ($p < 0\ 001$) in th 9 boys in whom it wa measu ed All the boy had in r a ed th ir max $\dot{V}_{O_2}$ by a mean value of 19 % ($p < 0\ 001$)

A larg part of th ob rved change an be a cribed to the growth which took place in th period betwe n the two xaminations In ord r to liminate thes chang s and ac ordingly to obtain a pictur of th chang s whi h could be ascribed to training th atio betw n valu s obtained after and before training r pectiv ly co rected fo body height was used (Table 14) Neither lung volume heart volum blood volume no total Hb then display d any increas Only total pota sium inc e ed by 6 % ($p < 0\ 01$) However weight d clined by 6 % ($p < 0\ 01$) Max $\dot{V}_{O_2}$ increa ed by 14 % ($p < 0\ 01$) and by 12 % ($p < 0\ 01$) for the 9 boys in whom total potassium had be n d termined

Co variation among the diff rent variabl was te ted with r gres i n analysis It wa then noted that the coeffi ients f co relation betwe n max $\dot{V}_{O_2}$ and the other variabl wer low before training There was signifi nt

Table 15 Correlation coeffici nts betw en variables befo e and afte physical training insert d in the equation. y ax^b Parenthesis indicates non significant r

y / x	Max $\dot{V}_{O_2}$	K	THb	HV	TLC	W
I BEFORE						
K	(52)					
THb	(48)	69				
HV	67	74	67			
TLC	(58)	74	69	97		
W	(42)	75	76	68	79	
H	(35)	8	72	88	87	70
II AFTER						
K	95					
THb	95	95				
HV	77	84	84			
TLC	83	90	92	94		
W	77	85	88	68	88	
H	81	90	92	87	93	84

corr lation only for heart volume All the coeffici nts of correlation wer h gher after training Coeffici nts of correlation fo max $\dot{V}_{O_2}$ with total potassium and total Hb w re specially high Th co relations were also better with h ight than w th weight (Table 15)

Since the logarithms of the measured valu were u ed for regression analyse t was possible to make omparisons betwe n the co ffi ients of regr ssion and th oret ally conc ivabl expon nts It was th n noted that ther was bett r g n ral ag eement with the anticipat d exponents after train ing Of all th regression co ffi i nt for height max V_{O_2} was found to have a value of 1 98 as compa ed to th theor t cal 2 0 Good ag em t was also found between total Hb (2 53) and h art olume (3 04) and th theor tical x ponent 3 There was fairly good agre ment for TLC (2 25) and weight (2 23) On the other hand total potassium had a oeffi nt of regr ssion of 1 38 a

oppo d to a theoretical valu of 0 67 This means that the subje ts were not scal models with respect to the muscula mass used for max $\dot{V}O_2$ The taller boys u ed a greater per c nt of their mu cular mass for max $\dot{V}O_2$ than the shorter boys

Afte training all coefficients of regr ssion displayed fa bett r agreement with the theoretical exponents and the co fficients of correlation were also highe This sugge ts that the condition of the boys after training bore greater r mblance to the normal state with espect to these physiological parameters

Comment

W ll trained athl te both adults and young people are cha a terised by max $\dot{V}_{O_2}$ values w ll co related to the larger d mensions of their oxygen transporting organs (B vegård 1962 Astrand et al 1963) Since training increas max $\dot{V}_{O_2}$ by 15 20 % co responding incre s e in th dimensions of the oxygen transporting o gans should be expected This was not the case for the p e nt mate ial The sam circumstance also appli d to Ekblom s study aft r 6 month of training (Ekblom 1969a) How ver an increa in the dimensions was noted in that tudy aft r 32 months of training

Preliminary proc ssing (Engström et al. 1971) of an ongoing l ngitudinal tudy of gi l swimme has di closed a signifi ant increas in pulmonary VC in relation to body length fo gi l who had continued to train at wimming fo eve al years Only a growth induced incr as could be d monstrated in the girls who had topped training

On hypothesis advanced ha uggest d that th physi al training of young people during their period of apid growth hould have a potentially greater ffect on th dimensions f th heart and lungs (A t and t al 1963 Ekblom 1969b Eng tröm et al 1971) Howeve the p e nt study was unable to produc support fo this hypoth i But a training period of 4 6 month might conc ivably be too bri f fo the ff ts to become manif st Howev r the wa also the dis repancy betw n th increas in functional capa ity of irculatory and respi tory o gans and the failure of the dim nsions of thes o gan to in reas Th re i a striking similarity in this respect to c rr pond ng examinations of adults (Saltin t al 1969)

In ontrast to th tuation with r pe t to the dimensions of the h art and lungs ther was a ignifi ant in a in muscular ma s Thi als provided the be t o relation with max VO_2 a circum tan which i ather s on abl sinc th mu culature is the body larg st con um of oxygen during xerci

Desp te the inc in mu cular ma s body weight only increa ed by 0 5 kg fo th 9 boys in whom total pota sium w s mea ured A 12 g increas

in total potassium corresponds to an increase in non adipose tissue of about 5 kg or 4 kg if most of the increase was in muscular tissue Thus there must have been a loss of more than 3 kg in adipose tissue during the period of training Th s means that more than 28 000 kcal (117 MJ) were metabo lized by the body s adipose tissue producing an altered body composition Similar results have be n obtained in the training of overweight boys (Sterky 1971)

Anaerobic en rgy delivery (V)

Alactacid anaerobic energy delivery take place by virtue of a breakdown of th energy rich phosphag ns adenosine triphosphate (ATP) and creatine phosphate (CP) The re ting concentrations of thes phosphag ns was 4 3 and 14 5 mmol s x kg ^{1} wet weight respectively During exercise the CP concentration gradually declined and was down to 4 4 mmoles x kg ^{1} wet weight immediately after maximal work; the ATP concentration was constant right up to maximal work levels when a slight decr ase was noted (Table 16) After training the ATP and CP concentrations had increased strikingly (Table 16) Exe cise produced changes similar to those not d prior to train ing but the drop after maximal exercise was somewhat greater Th con centrations immediately after work accordingly came to resemble those ob tained aft training (Table 16)

The lactate conc ntration in skel tal mu cle was 1 0 mmoles x kg ^{1} wet weight and incr a ed in exercise to 8 8 mmoles x kg ^{1} immediately after maximal exercise There wer similar values after training both at rest and at the same absolute submaximal work loads But there was a higher concentration in maximal work i e 13 7 mmoles x kg ^{1} wet weight (Tabl 16) The muscle lactate concentration before and after training was related to the oxyg n deficit m asured at the start of each respective work load The blood lactate oncentration eflected the muscle lactate concentration (Tabl 16)

Th glycog n content in muscle was 54 mmoles glucose units x kg ^{1} wet we ght Evaluated on the basis of PAS staining both fiber types contained equal amounts of glycogen A work load increased glycogen content gradual ly d reas d and was down to 33 mmol gluco e units x kg ^{1} wet weight aft r maximal ex rc se A 31 % high r r sting valu was noted aft r traini g and PAS staini g wa h avier fo both fiber type The drop in glycogen dur ing exercis wa gr ate after tra ning and the s me glycogen conc ntration was achi ved in maximal work as before training (Table 16) This ca partly be xplained by th fact that th work performed was omewhat h a ier after training Th mean maximal work load was thus 1 014 kpm x min ^{1} aft r

T bl 16 M an alu with SE fo mu cl metabolites ad no ine tripho phate (ATP) c ati pho ph t (CP) glycog n gluc glu o 6 pho phate (G 6 P) and lactate in muscl and blood at r t and du ing ex rci in 11 13 y old boy befo (B) and after (A) 4 *month* of phy i al t aini g Level of ignific nce fo pai ed valu btained before and afte phy ical trai i g xxx p < 0 001 xx p < 0 01 x p < 0 05 n p > 0 05

Condition	n		Blood lactate		ATP		CP		Glycog n glu ose units		Muscl lactat		Glucos		G 6 P	
kpm x min 1			mmol x1 1						mmoles x kg 1 wet w ight							
			B	A	B	A	B	A	B	A	B	A	B	A	B	A
At re t	6	Mean	0 8	1 2	4 3	4 8	14 5	20 2	54	71	1 0	1 5	0 5	0 6	0 2	0 4
		SE	0 1	0 1	0 1	0 1	0 8	0 8	3	3	0 2	0 2	0 1	0 1	0 1	0 1
		Sign	x		xx		xx		xx		n s		n s		x	
500	7	M an	2 3	2.1	4 3	4 8	9 3	14 7	51	58	3 0	3 3	0 9	0 9	0 6	0 9
		SE	0 3	0 3	0 3	0 3	1 1	1 9	5	3	0 5	0 7	0 1	0 2	0 2	0 1
		Sign	n		n		xx		n s		n		n s		n s	
750	6	M an	3 7	3 3	4 2	4 1	6 0	9 6	44	47	6 7	5 6	1 3	1 4	1 2	1 0
		SE	0 6	0 7	0 2	0 3	6 0	1 2	8	6	1 2	1 8	0 2	0 2	0 2	0 2
		Sign	x		n		n s		n s		n s		n s		n s	
Max exe cise 850 (B) 1014 (A)	7	M an	4 7	5 9	4 0	3 9	4 4	6 0	33	34	8 8	13 7	1 7	1 9	0 9	1 3
		SE	0 2	0 2	0 2	0 2	0 8	0 9	5	5	1 4	1 2	0 2	0 2	0 1	0 2
		Sign	xx		n s		n		n s		x		n s		n s	

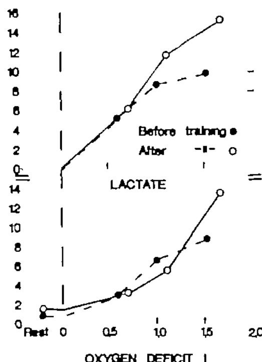


Fig 7 M an valu s for mu cle lactate and phosphagen deple tion in r lation to the oxygen deficit observed at the tart of xercis

training as compared to 850 kpm x min ¹ before training

Comm nt

In comparison to adult th boy displayed similar ATP and CP conc ntra tions at rest while glycogen content wa lower The drop in both phosphagens and glycog n during exercise was imilar to th situation in adults Muscl lactate conc ntration in both ubmaximal and maximal ex rci e was lower than fo adults (Fig 7) and this is in good agre ment with the low oxygen d ficit mea ur d The low muscle lactate concentrations may have been du to lower p oduction and/o f te m tabolism How v a lower la tat produ tion s the probabl cause a a rate limiting enzyme for glycolysis PFK displayed activ ty wh ch wa 40 % of the value for adults Another fa tor in support of th s vi w s th t ch ldr n have a lower capacity fo super maximal wo k

Compared to 13 14 y ar old boy (E ik on Karls on and Saltin 1971) these boys displayed s m l valu s fo mus l lactat at ubmaximal work lo ds (Fig 7) Thus an robı n gy supply is r stricted in children and youths as ompar d to adult

Aft r training there w a s gn f nt e in both ATP and CP An increas in ATP conc nt at on ha bee not d in the training of adults (I arlsson Diamant and Saltin 1971 F ls on et al 1972) Howe r an in

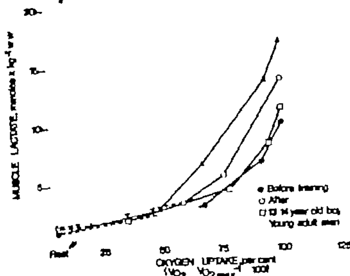


Fig 8 Mean mus l lactat concentration in r la tion to the oxygen uptake expr e ed as a per cent of the individual max VO_2 Included in the figure are data fo 13 14 year old boys (Eriksson Karlsson and Saltin 1971) and for adult m n (Karlsson Diamant and Saltin 1971 Knuttgen and Saltin 1972a and b)

crease in CP has only been found in animals (Ferdman and Feinschmidt 1929) Th CP concentration in boys was also latively high compared to th values for adults (Karls on 1971) However ther was a similar breakdown of CP and ATP during xercise after t aining and ther wa no d finite hange in the relationship betw en phosphagen breakdown and oxygen d ficit (*Fig 8*)

Mu lc lactate con ntrations wer identical at the same absolute submaximal work load afte training (T ble 16) This means that the muscle lactate c n ntration wa high r at th same lative work load before training This differentiates th boys from adults in whom a lower muscle lactate concentration was found at the same relative wo k load after training (Karlsson Diamant and Saltin 1971 Ka l son et al 1972) Accordingly the muscle lactate conc ntration after training in the 11 13 y ar old boys studied was higher at ompa able lo ds than th value obtained for 13 14 year old boys (Eriksson Karlsson and Saltin 1971) This do s not appea to indicate that th hange obtained afte training was du to bi logical maturation.

On th the hand th blood l ctate concentration was unchanged aft t aining even wh n the relative load is onsidered pointing to an incre sed lactate metabolism in th ti su or an incr ased lactate metaboli m the mu cl lls The increa ed oxidative capacity of the fiber with lo myo in ATP-as activity (ST fiber) supports this latter view

In his book "Growth at Adolescence (1962) Tanner analy ed th different factors controlling the growth of the body s different organs Among other things he emphasized how strongly genetic factors influence every change in organ size and function Diff rent exogenic agents such as disease mal nutrition and inactivity may inhibit natural development On the oth r hand there is some doubt as to whether factors exist which acc l ate d velopment

In general ev ry organ in the body is capable of a compensatory increase when required This compensatory increase occurs more rapidly in children than in adults probably because children are still growing Compensation is achieved by virtue of an increa e in growth rate The physical training of adults produces improved function for the oxygen transport organs There fore it is reasonable to question wh ther or not children increase their oxy gen transport capacity by mean of training mo e rapidly than adults

Howev r the fact that children are growing creates a special problem in the valuation of experimental results in this field In an unchanged environ m nt adults have a constant or only a very slowly declining physical work capacity Th results of a period of training can therefore be not d by a chang in the p eviously constant oxygen tran port capacity How ver oxygen transport capacity changes in the cours of morphological growth The eff ct exerted by any pe iod of training is th refore manifested as a greater increase in oxygen transport capacity than morphological growth alone might be x pected to produce

Th training of previously untrained adults increas s max $\dot{V}_{O_2}$ by 10 20 % (Rowell 1962 Saltin et al 1968 Ekblom et al 1968 Saltin et al 1969 Kilbom and Astrand 1971 and others) When the valu s obtained in these studi s are compared with results from trained children only the change in max V_{O_2} corrected for the influence of growth on max V_{O_2} during the t ain ing period can be used In th pr sent study the increase in max V_{O_2} during the period of t aining wh n corrected for th influenc of growth amounted to 14 " which is in ath r good agre m nt with th value found in adults Thus the process of growth doe not make children more receptiv to the nfluenc of training at the training ntensities used in this study

The reason why th hypothesi that children should increas their max $\dot{V}_{O_2}$ mor than adults was not conf med, is to be found in the diffe ent limit ing factors aff cting erobic capacity Since max $\dot{V}_{O_2}$ is a fun tion of maxi mal heart rate maximal stroke volum and maximal (a $\bar{v}$)O_2 difference this m ans that these mutually riable p ramet rs limit max $\dot{V}_{O_2}$ Maximal heart rat is somewhat high r in hild n than in adults but ther ar g eat inter individual differences (Robin on 1938 Ast and 1952) The training of

adults actually lowers maximal hea t rate This was also noted for the boys in the p sent study

Cardia stroke volume is cl arly small r in childr n than in adult mal s but wh n xpress d on the basis of height cubed th y are similar (Tabl 12) A pronounced incr as in stroke volume was noted for the boys after t aining This increa e was g eat r than that which occurs in adults (Table 12) The final facto maximal (a $\bar{v}$)O_2 diffe ence is governed among other things by the blood s Hb concentration The Hb concentration i lower in children than in adults (Tanner 1962) which mean that the blood s O_2 binding capacity i acco dingly lowe The Hb con entration was unchanged after the period of physical training Thus there was no increa in maximal (a $\bar{v}$)O_2 diff r enc by means of this factor This indicates that the distribution of cardiac output to the working muscl s or any additionally nhanced extraction of oxy gen from th arterial blood flow of the musculature was not obtained

Thus the ntir increas in th boys max $\dot{V}_{O_2}$ was the re ult of increased stroke volume This di tingui hes boys from adult males who also display an incre s in maximal (a $\bar{v}$)O_2 diff renc after a short period of training (Rowell 1962 Ekblom et al 1968 Saltin et al 1968) In this manner the increa in max $\dot{V}_{O_2}$ is no gr ater than fo adult despite the boys greater increas in troke volume The hypothesi that th aerobic capacity in child en hould increas mo e following training than in adults wa not confirmed

On the other hand a large aerobi capacity at an adult age p sume a la g troke volum (heart volume) If phy ical training is begun a early a in p puberty a larger stroke volume (heart volume) may be attained in adult hood than if training were initiated late in lif This hypoth is has yet to be nfi med But th troke volume increas ob rved in this study sugg ts that this hypothesi may be valid The la ge heart volum s in young top athletes (Astrand t al 1963) al o provid indi t support fo that hypoth si Ekblom (1969 a) was also abl to not a signifi antly incr a ed heart volum in 11 ye old boys afte 32 months of training This was also tru when th ffe t of growth was onsid red

With pect to the r lationship between stroke volume and heart volum studie from adults have hown that the incr ase in strok volume very often fails to produc a co ponding increase in heart volume Thi was also th cas f r the boys in the p nt study The ason fo thi i not known A edu tion in th nd ystolic volume of th heart hambe s ould be on x planati n but no tudie on man have been pe formed on training to d mon trate that t aining has any such eff ct (Mit hell and Wild nthal 1971 Ro kamm and Reind ll 1972) If this xplanation i o ct it means that there an be no furth edu ti n in nd sy tolic volume if training ontinue f a long period of time and that the incre d stroke volume must aris by mean of

a simultaneous increase in heart volume Support for thi vi w is provided by th high correlations that existed betw en max V_{O_2} and heart volum in well trained athletes at early ages (Astrand et al 1963)

There wer great difference betw n children and adults with respect to anaerobic capacity (Astrand 1952 Karlsson Diamant and Saltin 1971) The reason fo th diff r nces in both blood and muscle lactate in submaximal and maximal xerc se has been the subject of many hypoth ses such as a reduc d ability to form lactat a greater rate of intra and ext amuscular metaboli m and a great r l vel of dilution because of the relativ ly higher total water content in children PFK has been claimed to be the rate limiting enzyme for the glycolysis (Danforth and Lyon 1964) The low r values for PFK and muscle lactat during work support their finding It is not known why hildren possess this limitation It wa not uncommon to find the corr s ponding oxidative enzyme in concentrations equal to th adult level

Little is known about the facto s involved in this PFK concentration in crease to adult level or when the increas tak s place during growth Howeve the increase is not exclus vely due to biological maturation The results of the pres nt study suggest that physical training may play an important role (Table 7)

The view that physical training has a local ffe t with re pe t to glycolytic capacity and also leads to a probabl incr ase in intra and extramuscular lactate m tabolism is upported by the circumstan e that muscle lactate con c ntration after training wa higher at th sam relative work load (Fig 8) than befo e training in contrast to blood la tate conc ntration Both the in creased SDH activity and th increas d intensity of DPNH-diaphorase staining suggest that the muscle cell had acquired greater aerob capac ty

For hund eds of yea s children wer gard d as scaled down copi s of adults Dis over s n th past d ade have shown that there are striking qualitati e differences betw en children and adults Reduced lactacid anae ob ap ty is one such diffe ence In daily lif such a diff rence has limited practical sign f cance but it may become important in physi al x r cis and sports Thus childr n should be capable of d aling with very brief int nse xe cise pi od s n which primar ly alactacid anaerobic metabolism is nvolved i e pho phagen d pletion How ver l ss intensive exercise in wh h the durat n of work is more than 20 to 30 sec the energy d livery through glycolys s be om s mo e mportant Th childr n then do not perform as well as adults If th du ation of exercise is longer than 4 5 min a robi metabolism becomes r ponsibl fo most of the ne gy deliv ri s and th re is no apparent diff r nce b twe n hildr n and adults These results a e in very close agreement with the p actu al xperienc alr ady gained by swimming trainers Swimm ng th port in whi h young individuals can

achieve top results How ver a young swimmer usually first reach s the top at the long distanc s (400 1500 m) at which th duration of xercis i longer than 4 min They then tend to g t better at th shorte lactat d manding distances as th y g t old r

Tanner s afor mentioned view on the genetic control of organ growth and function appea to requi e modification with r spect to function judgi g by the r sults of the p es nt study Before training mo phological and functio al variabl failed to display co va iation in the manner anticipated on th basis of a dimension analysis vi w Much botto ag ement with the anticipated co variation was obs rved aft r the training period This an only be interpr ted to m an that a c rtain d gree of physical exerci is necess ry fo functional ly harmonius somatic development and that the boys tudied had a level of physical activity before t aining which was l ss than the minimum l vel r quired

This leads to two conclusions one theoretical and one p ctical Maximal oxygen uptake is a somatic function whos development in lation to omatic development in othe respects is controlled by both genetic facto s and by an xogenic factor i the d g of physical activity The practical co sequ nc of this mu t be that growing individual in urbani ed industrialis d societi s must be given a suffici nt amount of phy ical t aining Growing young peopl must be provided with incr ased phy ical training both du ing school hours and during thei pare time and it i important for the com munity to allocate re ourc fo this purpos

SUMMARY

Twelve boy 11 13 yea from a Sto kholm suburb wer trained th ee time a we k for four months Their max V_{O_2} increased by 19 % to 2 21 l x min 1 o sponding to a 16 % increa e to 48 5 ml x kg 1 x min 1 per kg of body weight and to a 14 % inc eas to 0 92 l x min 1 x m 2 pe height qua ed Thi inc as in max V_{O_2} was no g ate than the value attainable in adult with rr ponding training H art volume increased by 10 % to 544 ml TLC increa ed by 7 % to 3 84 l VC inc a ed by 4 % to 3 14 l but other static and dynami pulmonary volum were unchanged Total Hb i creased by 10 % to 428 g blood volum inc d by 12 % to 3 28 l and total pota sium inc ased by 14 % to 95 3 g During the training period the boys g ew 3 5 m but th wa no ignifi ant in as in weight Wh n correction f the ff t of growth wa mad there wa only an increas in total pot um (6 %) and max V_{O_2} (14 %) weight declining by 3 %

V_T/VC V_E/V_{O_2} and $\dot{V}_A/V_{O_2}$ before t aining were somewhat l than fo

adults in maximal exercise but this difference was not found after training $D_{L_{CO}}$/FRC in near maximal exercise was somewhat lower than for adult men and training produced no increase $P_{A_{O_2}}$ $P_{a_{O_2}}$ at rest and in submaximal exercise was somewhat lower than for adult m n There was a higher $P_{A_{O_2}}$ $P_{a_{O_2}}$ in submaximal work after training In maximal exercise $P_{A_{O_2}}$ $P_{a_{O_2}}$ was 22 mm Hg and training produced no change

Cardiac output in submaximal work both before and after training was 1 2 l less than for adult men and the (a $\bar{v}$)O_2 difference was correspondingly higher Maximal cardiac output was 12 5 l x min ^{1} before training and 14 6 l x min ^{1} after training; stroke volume was 67 ml before and 80 ml aft r training The maximal (a $\bar{v}$)O_2 difference was 14 2 ml x 100 ml ^{1} and there was no increase after training

The boys had a lower anaerobic capacity than adults Thei maximal values for oxygen deficit and blood and muscle lactate concentrations were 1 48 l 8 0 mmoles x l ^{1} and 8 8 mmoles x kg ^{1} wet weight respectively The corresponding values after training were 1 64 l 9 5 mmol s x l ^{1} and 13 7 mmoles x kg ^{1} wet weight r spectively Their alactacid anaerobic capacity was not lower than in adults ATP concentration was 4 3 mmoles x kg ^{1} wet w ight and CP concentration 14 5 mmoles x kg ^{1} w t weight in th musculatur Aft r training there we e trikingly higher concentrations i e 4 8 and 20 2 mmoles x kg ^{1} wet weight respectively Glycogen concentration was also somewhat higher after training In adults there is a d clin in both muscle and blood lactate concentrations in submaximal exer ise aft r training This was not the case for the boys who displayed higher muscle lactate con c ntrations their blood lactate concentrations were unchanged

Five oth r 11 2 year old boys from the same school trained on a bicycle ergometer 3 t mes a week for 6 we ks for at l ast 20 min per s ssion at a work load corresponding to at least 70 % of their max V_{O_2} Th y increased their max V_{O_2} by 6 % while SDH activity incr ased by 30 % to 7 0 µmoles x (g x min) ^{1} But PFK activity i e 8 4 µmol s x (g x min) ^{1} was less than 50 % of adult value Training produced an 83 % increase in PFK activi ty The low PFK activity may be one explanation of the boys low lactacid anae obic capacity Th population of fast twitch glycolytic fibers (FT) and slow twitch oxidative fibers (ST) was the ame as in adults and training produced no chang

REFERENCES

ASTRAND I Aerobic work capacity in men and women with special reference to age Acta physiol scand 1960 49 Suppl 169

ASTRAND P -O *Experimental studies of physical working capa ity in relation to sex and ag* Copenhag n Munksgaard 1952

ASTRAND P -O and K RODAHL T xtbook of wo k phy iology New York Mc Graw Hill Book Company 1970

ASTRAND P -O T E CUDDY B SALTIN and J STENBERG Cardiac output du ing submaximal and maximal work J appl Phy iol 1964 19 268 274

ASTRAND P -O L ENGSTRÖM B O ERIKSSON P KARLBERG I NYLANDER B SALTIN and C THORÉN Gi l swimmers Acta paediat (Uppsala) 1963 Suppl 147

BAGGLEY G and G R CUMMING Serial measur ment of working and *erobic apacity of Winnipeg chool children du ing a year* Int Environ mental ff cts on wo k performance d G R Cumming D Sindahl and A W Taylor 1972 173 186

BARKER S B and W H SUMMERSSON The olorimetric determination of lactic acid in biologi al material J biol Chem 1941 138 535 554

BARNARD R J V R EDGERTON and J B PETER Effect of xercise on skel tal mu l I Bioch mi al and hi t chemical properties J appl Phy iol 1970 28 762 766

BAR OR O J S SKINNER V BERGSTEINOVA C SHEARBURN D ROYER W BELL J HAAS and E R BUSKIRK Maximal aerobi capacity of 6 15-y ar old girls and boy with ubno mal intelligenc quoti nt A ta p diat and 1971 60 Suppl 217 108 113

BAR-OR O and L D ZWIREN Physiological effe ts of increased frequ ncy of phy i al edu ti n l and of ndu anc conditio ing on 9 10 ye ld gi ls and boys Pro 4th Intern ymp Paediat W k Phy iol Wing t In titut I 1 1972

BENGTSSON E The working apa ity in normal child n valuated by submaximal xe ci n the bicycle g meter and compared with adults Acta med and 1956 154 91 109

BERGGREN S M The oxyg n deficit of arterial blood caused by non venti lating parts of th lung Acta physiol scand 1942 4 Suppl 11

BERVEN H The physical working capacity of healthy children Acta paediat (Uppsala) 1963 Suppl 148

BEVEGÅRD S Studies on the regulation of th circulation in man With special reference to the stroke volume and the effect of muscular work body position and artificially induced variations of the heart rate Acta physiol scand 1962 57 Suppl 200

BJURE J G GRIMBY and N J NILSSON Pulmonary gas exchange during submaximal and maximal exercie in healthy middle aged men In: Pul monary diffusing capacity on exercise ed M Scherrer Hans Huber Publishers B rn Stuttgart Vienna 1971 107 131

BROMAN B G DAHLBERG and A LICHTENSTEIN Height and weight during growth Acta paediat (Uppsala) 1942 30 1-66

COMROE J H Jr R E FORSTER A B DUBOIS W.A BRISCOE and E CARLSEN The lung Clinical physiology and pulmonary function tests 2nd Ed Year Book Medical Publishers Chicago 1967

DANFORTH W H and J B LYON Jr Glycogenolysis during tetanic con traction of isolated mouse muscles in the presence and absence of phos phorylase a J biol Chem 1964 238 4047-4052

DANIELS J and N OLDRIDGE Changes in oxygen consumption of young boy during growth and running training Med sci Sports 1971 3 161 165

von DÖBELN W and B LINDELL Som aspects of radon contamination following ingestion Arkiv Fysik 1964 27 531 572

EKBLOM B Effect of physical training in adolesc nt boys J appl Physiol 1969a 27 350 355

EKBLOM B Eff ct of physical training on oxygen transport in man Acta physiol scand 1969b Suppl 328

EKBLOM B and L HERMANSEN Cardia output in athlet s J appl Physiol 1968 24 619 625

EKBLOM B P -O ASTRAND B SALTIN J STENBERG and B WALLSTROM Effect of training on c rculatory respons to exercie J appl Physiol 1968 24 518 528

ENGSTROM I B O ERIKSSON P KARLBERG B SALTIN and C THORÉN Preliminary report on the d velopment of lung volumes in young girl swimmers Acta paediat scand 1971 60 Suppl 217 73 76

ERIKSSON B O P D GOLLNICK and B SALTIN Unpublished results 1972

ERIKSSON B O G GRIMBY and B SALTIN Cardiac output and arterial blood gases during exercise in pube tal boys J appl Physiol 1971 31 348 352

ERIKSSON B O J KARLSSON and B SALTIN Muscl metabolites during exercise in 13 year old boys Acta paediat scand 1971 60 Suppl 217 57 63

FERDMAN D and O FEINSCHMIDT Uber den Einfluss d s Trainier ns des Muskels auf s inen Gehalt am Phosphorverbindung n Hoppe Seylers Z physiol Chem. 1929 183 261 268

FILEY G F D J Mac INTOSH and G W WRIGHT Carbon monoxide uptake and pulmonary diffusing capacity in normal subj cts at rest and during exer i J lin Invest 1954 33 530 539

GOLLNICK P D R B ARMSTRONG C W SAUBERT K PIEHL and B SALTIN Enzyme activity and fiber composition in skeletal muscle of untrained and trained men J appl Physiol 1972 33 312 319

GRANATH A B JONSSON and T STRANDELL Circulation in healthy old men tudied by right h art catheterisation at rest and during exercise in supine and sitting position Acta med cand 1964 176 425 446

GRIMBY G R spiration in exercis Med ci Sports 1969 1 9 14

GRIMBY G N J NILSSON and B SALTIN Cardi output during sub maximal and maximal xercis in activ middle aged athletes J appl Phy iol 1966 21 1150 1156

HARTLEY L H G GRIMBY A KILBOM N J NILSSON I ASTRAND J BJURE B EKBLOM and B SALTIN Physical training in sedentary middl ag d and old r men III Cardiac output and gas exchange at ub maximal and maximal xerci Scand J lin Lab Inve t 1969 24 335 344

HERMANSEN L Ana robi ene gy rel as Med s i Sports 1969 1 32 38

HERMANSEN L and S OSEID Di t and indir ct stimation of maximal oxygen uptake in pr pubertal boys Acta paediat s and 1971 60 Suppl 217 18 23

HERMANSEN L and B SALTIN Oxyg n uptake during maximal tre dmill and bicycle xercis J ppl Physiol 1969 26 31 37

and training Circulation 1968 38 Suppl 7

SCHOLZ R H SCHMITZ T BUECHER and J O LAMPEN Uber die Wirkung von Nystatin auf Bäckerhefe Biochem Z 1959 331 71 86

SELIGER V V CERMAK P HANDZO J HORAK Z JIRKA M MACEK M PRIBIL J ROUS O SKRANC J ULBRICH and J URBANEK Physical fitness of the Czechoslovak 12 and 15 year old population Acta paediat scand 1971 60 Suppl 217 37-41

SHEPHARD R J C ALLEN O BAR-OR C T M DAVIES S DEGRE R HEDMAN K ISHII M KANEKO J R LaCOUR P E di PRAMPERO and V SELIGER The working capacity of Toronto school-children Canad med Ass J 1969 100 560 566

SIGGAARD ANDERSEN O K ENGEL K JØRGENSEN and P ASTRUP A micromethod for determination of pH carbon dioxide tension base excess and standard bicarbonate in capillary blood Scand J clin Lab Invest 1960 12 172 176

SJÖSTRAND T A A method fo the determination of the total hemoglobin content of the body Acta physiol scand 1948 16 211 231

SNEDECOR G W Statistical methods 6th ed The Iowa Stat University P es Amer Iowa 1967

SPRYNAROVA S Development of the relationship between aerobic capacity and the circulatory and r piratory reaction to mod rate activity in boys 11 13 years old Physiol bohemoslov 1966 15 253 264

STERKY G Clinical and metabolic aspects on obesity in childhood In: Muscle m tabolism during exercise ed B Pernow and B Saltin Plenum Press New York 1971 521 527

STRÖM G The influence of anoxia on lactate utilization in man after pro long d muscular work A ta physiol scand 1949 17 440-451

TANNER J M Growth at adoles ence Blackwell Scientific Publications Oxford 2 ed 1962

THOREN C Unpubl shed sults 1967

KUNGSBACKA 1972
ELANDERS BOKTRYCKERI AKTIEBOLAG

ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 385

MUCOSAL BLOOD CIRCULATION AND ITS INFLUENCE ON PASSIVE ABSORPTION IN THE SMALL INTESTINE

An experimental study in the cat

BY

JOAR SVANVIK

GÖTEBORG 1973

Department of Physiology University of Göteborg Göteborg Sweden

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 385

MUCOSAL BLOOD CIRCULATION AND ITS INFLUENCE ON PASSIVE ABSORPTION IN THE SMALL INTESTINE

An experimental study in the cat

By

Joar Svanvik

Göteborg 1973

CONTENTS

This summary is based on studies reported in the following papers.

I An indicator-dilution method for studying intestinal haemodynamics in the cat
B Biber O Lundgren L Stage and J Svanvik. Acta physiol scand 1973 In press

II Intramural blood flow and blood volume in the small intestine of the cat as analyzed by an indicator-dilution technique
B Biber O Lundgren and J Svanvik. Acta physiol scand 1973 In press

III Mucosal haemodynamics in the small intestine of the cat during reduced perfusion pressure
O Lundgren and J Svanvik. Acta physiol scand 1973 In press

IV Mucosal haemodynamics in the small intestine of the cat during regional sympathetic vasoconstrictor activation
J Svanvik. Acta physiol scand 1973 In press

V The influence of blood flow on the rate of absorption of ^{85}Kr from the small intestine of the cat
B Biber O Lundgren and J Svanvik. Accepted for publication in Acta physiol scand

VI The effect of reduced perfusion pressure and regional sympathetic vasoconstrictor activation on the rate of absorption of ^{85}Kr from the small intestine of the cat
J Svanvik Accepted for publication in Acta physiol scand

The papers are referred to by their Roman numerals in the text

INTRODUCTION

The two important physiological functions of the small intestinal mucosa are absorption and secretion. Absorption takes place across the epithelial cells lining the luminal surface while secretion is supposed to occur mainly in the crypts. In the light of present knowledge the small intestinal mucosa is a structure of relatively low passive permeability but containing a variety of highly specific transfer systems for biologically important substances. However, the concept that the intestine absorbs almost everything present in the lumen in a diffusible form, independent of the body's needs, nevertheless holds true and normally a variety of such substances enter the lumen. Thus absorption is partly "active", dependent on the metabolism of the absorbing cells and partly "passive", controlled by the law of diffusion. Secretion is presumably also an "active" process that calls for an adequate supply of oxygen. The nutritional demands of the intestinal mucosa are also high, partly due to the rapid turnover rate of the epithelium where the total cell population appears to be replaced in about two days. A rapid cell renewal therefore occurs, probably in the crypts from which the cells are gradually displaced towards the villous tips.

The mucosal blood supply serves the nutritional demands of the tissues outlined above. There are, however, reasons to believe that the intestinal mucosa is often considerably overperfused in relation to its own nutritional oxygen demands, partly because mucosal blood is the transport vehicle for most of the absorbed substances and also partly because plasma is the raw material for secretion.

The intricate morphological features of the small intestinal mucosa include a complex vascular arrangement (vide infra) with separate circuits supplying the villi and the crypts. The functional consequences of this peculiar vascular design seems to be even more complex. Thus it was recently shown that a countercurrent exchange of diffusible solutes occurs between the ascending and descending vessels in the villi (see Lundgren 1967). Due to the presence of this exchanger, easily diffusible substances injected intraarterially are shunted extravascularly from the arterial to the venous end of the villous hairpin vascular loop, diffusing across the vascular walls and the narrow tissue spaces. It was also demonstrated that lipid soluble substances were more easily "trapped" than water soluble ones, due to their different capillary permeability, in the exchanger thus created. Further, by increasing the intestinal blood flow and hence decreasing the time available for exchange diffusion, the efficiency of the countercurrent exchanger could be decreased. It was proposed that the existence of this countercurrent system would affect the intestinal

absorption and probably to a certain extent delay or even hinder net blood transport of absorbed substances The system would be relatively selective in the sense that those substances which most easily pass the intestinal epithelium (lipid soluble or low molecular weight substances) would also be most efficiently trapped in the mucosal countercurrent exchanger

Furthermore the characteristic vascular architecture of the intestinal mucosa-submucosa probably constitutes the anatomical prerequisite for the considerable plasma skimming that seems to occur in the intestine (Jodal and Lundgren 1970 a) The mucosal arterial vessels branch off more or less at right angles from the vascular network in the submucosa and/or in the deeper parts of the mucosa and these points offer a favourable situation for plasma skimming when flow rate is high enough Accordingly the superficial mucosal layers seem to be perfused by blood with an hematocrit only 50–60 per cent of the arterial hematocrit

The functional implications of the countercurrent exchanger as regards intestinal absorption have so far not been subject to any experimental test Moreover most if not all investigators studying the relationship between blood flow and rate of absorption in the intestine have recorded total intestinal blood flow (for ref see V and VI) due to a lack of appropriate methods for studying mucosal hemodynamics selectively When approaches such as various accumulation techniques have been employed they have allowed only one measurement per animal (for ref see I) An inert gas washout technique that allows repeated determinations was recently utilized for a study of blood flow in the different layers of the intestinal wall (Lundgren 1967) However the use of such easily diffusible tracers is for such purposes greatly complicated by the above mentioned extravascular transit of tracers between venous and arterial vessels in the intestinal mucosa

The present series of experiments (I–IV) represents an attempt to develop a technique for studying quantitatively the blood circulation in the mucosa of the cat small intestine using tracers that remain within the vessels thereby avoiding any extravascular passage of tracer in the countercurrent exchanger The technique involves a close intraarterial injection of intravascular tracers (labelled with ^{32}P or ^{198}Au) which are traced with β-sensoring devices placed in the intestinal lumen Since the volume of the region monitored by the detector is determined by the energy level of the β-radiation ^{32}P-labelled red cells and plasma colloids were monitored from almost all the mucosa while the ^{198}Au labelled plasma colloid particles were registered only from the villi Paper I deals with the theoretical background for the interpretation of the indicator-dilution curves

registered with the intraluminar detectors It was shown in this study that mucosal (villous) blood flow could be estimated from the maximal height of the curve mucosal (villous) blood content from the area under the curve and mean transit time within the monitored tissue volume from the area under the curve divided by the maximal height of the curve In the following reports (II-IV) this method was used for the study of small intestinal mucosal (villous) blood circulation in different hemodynamic situations

Knowing the hemodynamic features of the mucosal vascular bed it was thought relevant to investigate the relationship between mucosal blood flow and rate of absorption of a passively absorbed tracer For this purpose the easily diffusible inert gas ^{85}Kr was chosen as the first test substance since it had been used in earlier investigations on the gut and was known to be extravascularly shortcircuited in the mucosal countercurrent exchanger after i a administration (see Lundgren 1967) Thus it was regarded of interest to study the same substance in its passage from lumen to the intestinal blood stream and to estimate the earlier proposed trapping effect of the countercurrent exchanger

The absorption rate of this tracer was determined in experiments where the tracer luminal concentration of ^{85}Kr dissolved in saline was kept essentially constant The experiments were performed under conditions similar to those earlier investigated with the indicator-dilution technique (II-IV) Thus the absorption rate during resting conditions and hyperemia was studied in paper V and during reduced perfusion pressure and stimulation of the regional sympathetic nerves in paper VI

Preliminary reports of parts of this series of experiments have previously been published (Lundgren and Svanvik 1968 Biber Lundgren and Svanvik 1969)

Vascular arrangements in the intestinal submucosa and mucosa

The vascular morphology in the submucosa and mucosa of the small intestine has been studied by several authors in various animals The techniques that have been used usually include i a infusions of opaque material and fixation of the tissue prior to microscopic inspection and thus the accuracy depends on a complete filling of the vascular tree Furthermore, the submucosa and the mucosa except the villi have been investigated by intravital microscopy (Baez 1959) Differences in methodology are probably mainly responsible for the differences in the results obtained but there does seem to be considerable morphological variation between species as pointed out by Noer (1943)

The submucosa receives vessels that obliquely pass the muscularis coat from the serosa forming a dense plexus of arteries and veins which seems poor of capillaries (Mohiuddin 1966) Mall (1888) in the dog and Spanner (1932) in the cat, describe a characteristically structured arterio-venous anastomosis (Venenbällchen) This structure could however not be recognized in the rat by Baez (1959) or Mohiuddin (1966) Similar arterio-venous communications have been described in the small intestine of man but they seem to be more frequent in the gastric wall (Boulter and Parks 1959) The functional significance of these structures seems so far not to have been fully established and no large arterio-venous vessels were demonstrated in dog experiments with i a injected microspheres (Delaney 1969) Neither could Dresel et al (1966) find any evidence of true blood shunting in the cat intestine in the sense that any sizable portion of the blood could be excluded from nutritional exchange

In all cases the arterial vessels emanating from the submucosa supply two capillary networks in the mucosa one located around the crypts and one in the intestinal villi A great number of studies on man dog cat and rat point to separate arterial vessels from the submucosa to these two capillary networks (Heller 1872 Mall 1888 Jacobson and Noer 1952 Mohiuddin 1966) thus providing the basic conditions for a separate control of blood supply to the tissue around the crypts and to the villi A schematic illustration of this vascular anatomy is shown in Fig 1

The tissue around the mucosal crypts is provided by a dense capillary network supplied by numerous small arteries from the submucosa (Heller 1872 Mall 1888) This network seems denser in the jejunum than in the ileum (Reynolds Brim and Sheehy 1967)

The villi as pointed out above seem to be provided with separate arterial vessels from the submucosa In the dog Mall (1888) describes these arterial vessels as penetrating the muscularis mucosa and then dividing into 8–10 branches each supplying one villus and losing their smooth muscle coat at the villous base Although different studies disagree as to the arrangement of the arterial and venous vessels in the villi all authors describe a descending mono-layered capillary network in close contact with the epithelial cells Electron microscopy has shown that these capillaries have fenestrations with a diameter of 500 Å facing the epithelial cells often covered by a thin basement membrane (Horstman 1966 Clementi and Palade 1969 Casley-Smith 1971) The arterial supply to this network is described by most authors as a non-branching arteriole lacking smooth muscles and connected to the capillaries at the villous tip (Heller 1872 man dog cat and rabbit; Mall 1888 dog, Nisioka 1927 cat; Sponner 1932: man, dog and cat; Jacobson and Noer 1952: man dog and rabbit; Mohiuddin 1966 rat) In the cat the drainage from these capillaries occurs via veins in the villous base (Heller 1872 Nisioka 1927) although in man dog and monkey there seem to be veins in the upper part of the villus (Heller 1872 Mall 1888, Jacobson and Noer 1952 Reynolds and Swan 1972) conveying the blood to the submucosal plexus

A schematic illustration of this vascular anatomy is shown in Fig I In plate A B and C are seen sections of a cat s vasodilated jejunum where the tissue is cleared according to Spalteholz (1888) after in vivo infusion of India ink into the superior mesenteric artery

Nerve supply of the intestinal submucosal and mucosal blood vessels

The arteries and arterioles in the wall of the small intestine are densely innervated by adrenergic nerve fibres while the venous vessels are most sparsely supplied (Norberg 1964 Jacobowitz 1965) Thus, the arterial vessels in the submucosa and in the mucosa particularly in the layer between the muscularis mucosa and the base of the intestinal crypts have a rich innervation while the number of adrenergic nerves decreases progressively as the lumen is approached (Silva Ross and Osborne 1971) Since the small arterial vessels supplying the villi soon lose their smooth muscles their constrictor fibre supply must be concentrated to the most proximal parts where they emerge in the deepest mucosa

Cholinergic fibres around arteriolar vasculature are sparse but acetylcholinesterase staining fibres are numerous in the lamina propria in the villi (Jacobowitz 1965) The function of these latter fibres however is not clear

Fig 1 A schemati illustration of the vascular anatomy of a cat's jejunal mucosa For details see text Squares refer to roughly corresponding sections seen in plate A B and C

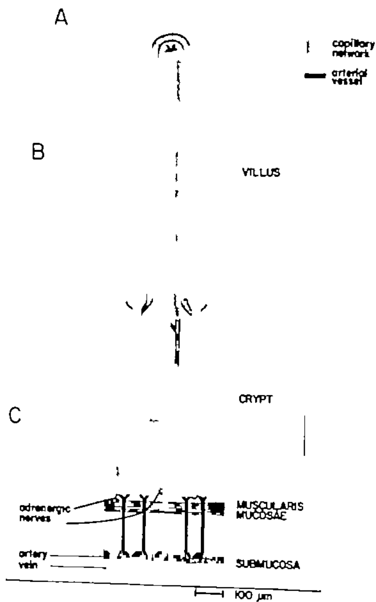
Plate A The villous vascular bed of a cat's vasodilated jejunum The ascending arterial vessel can be seen in the middle villus and the subepithelial capillary networks with their numerous cross connections, are also visible

Plate B The vascular anatomy at the base of the villi in a cat's vasodilated jejunum An ascending villous arterial vessel can be seen in the middle of the picture The villous capillaries can be seen to collect into veins in the middle and lower part of the picture

Plate C The vascular anatomy at the mucosal crypts and the submucosa in a cat's vasodilated jejunum Coarse vessels belonging to the submucosa are seen in the middle part of the picture In the lower part vessels of muscularis proper and serosa are seen

Plates A B and C After in vivo infusion of India ink into the superior mesenteric artery at intestinal vasodilatation (isopropylnoradrenaline) the tissue was treated according to Spalteholz (1888) x 100–150 The discontinuity of some vessels is due to their running out of focus

The plates are reproduced in collaboration with Drs Elof Eriksson and Rhagnar Myrhage Laboratory of Experimental Biology / Chief: Professor P -I Brånemark / Department of Anatomy University of Göteborg



A
capillary network
arterial vessel
B
VILLUS
CRYPT
C
adrenergic nerves
artery
vein
MUSCULARIS MUCOSAE
SUBMUCOSA
100 μm

FIG 1



PLATE A.



PLATE B



PLATE C

GENERAL METHODOLOGY

Observations were carried out on 110 cats anesthetized with chloralose i v (40-70 mg/kg b w) after ether induction The cats had been deprived of food for at least 24 hours and had no obvious signs of intestinal infection The isotope methods used for the blood circulation studies and the investigations on absorption are fully described in paper I and V respectively and will be discussed also in connection with the results (vide infra) A presentation of general procedures is given below

Operative procedures

The operative procedures used were similar to those of previous studies of intestinal blood circulation (cf Folkow, Lundgren and Wallentin 1963 Kampp Lundgren and Sjöstrand 1968) and were except for minor details identical in studies I-VI

The abdomen was opened in the midline and the greater omentum and the spleen were extirpated The spleen was electrically stimulated to expell its blood before extirpation A segment of the jejunum weighing 10-30 g was isolated and the remainder of the intestinal tract removed The lumen of the jejunal segment was flushed with bodywarm saline or Tyrode s solution until the effluent was clear The mesenteric vein which in a preparation like this drains all the blood from the intestinal segment and its lymph nodes was cannulated after heparinisation and connected to a drop recorder unit operating an ordinate writer The blood was returned to the animal via a funnel connected to the jugular vein Venous outflow pressure was set at a control level of about 10 mm Hg by adjusting the height of the tube draining the drop recorder Mean arterial blood pressure was recorded from the left femoral artery by a Statham pressure transducer Small branches of the superior mesenteric artery were cannulated to permit close arterial injections or infusions to the intestinal preparation or for registration of the arterial inflow pressure to the small intestine

The prevailing influence of the autonomic nervous system was eliminated by giving atropine i v (1-1 5 mg/kg b w) cutting the splanchnic nerves bilaterally and by denervating the left adrenal gland and by exclusion of the right one from the circulation by ligatures

Bleeding was carefully avoided during the operative procedures and if occurring substitution was performed by i v infusions of bodywarm Dextran-Tyrode solution

During radioactive measurements the intestinal segments were placed outside the abdomen on a lead plate covered with gauze soaked

by bodywarm saline and in turn covered by plastic film The tempera ture of the intestinal tissue was continuously controlled with a thermocouple thermometer placed in the lumen or on the serosa of the gut

To induce graded vasodilatations in the intestinal segment a constant i a infusion of isopropylnoradrenaline was made via a thin catheter in a small branch of the superior mesenteric artery Arterial inflow pressure to the intestinal segment which could be reduced by means of an adjustable clamp around the superior mesenteric artery was registered from a small branch to this artery Venous outflow pressure in the intestinal segment could be changed from the control level of about 10 mm Hg by adjusting the height of the tube draining the drop recorder Electrical stimulation of the regional sympathetic nerves were performed via a bipolar silver electrode mounted on the peripheral ends of the divided nerve fibres surrounding the superior mesenteric artery

At the end of each experiment the weight of the intestinal preparation was determined for calculations of blood flow and absorption rate per unit tissue weight

Protection from radioactivity

The handling of the concentrated radioactive solutions was carried out manually behind a lead glass window When ^{32}P and ^{198}Au were used rubber gloves (material thickness 0 5 mm) were worn During experiments recorded background radioactivity was kept low by covering syringes and reservoirs containing radioactive tracer solutions with lead plates All personel continuously working with these radioactive experiments were wearing individual dosimeter plates

BLOOD CIRCULATION IN THE SMALL INTESTINAL MUCOSA

The parallel-coupled vascular circuits in the wall of the small intestine have been studied by means of various accumulation techniques In most investigations by means of diffusible tracers (for ref see paper I) However, these methods do not give any reliable flow values for the mucosal circulation and particularly not for the villous circulation since the i a administered diffusible tracers will partly be excluded from the villi due to the mucosal countercurrent exchanger (Lundgren 1967 Kampp Lundgren and Sjöstrand 1968) An indicator-dilution method which is not influenced by the mucosal countercurrent exchange was therefore developed for a quantitative and separate study of the villous circulation and also of the entire mucosal circulation The theoretical background and the experimental procedures for this method were described in paper I A short presentation of the method and some methodological aspects are given below

Methodological considerations

The present method was developed to allow quantitative estimations of the mucosal circulation and particularly of the villous circulation which are of prime importance for intestinal function with respect to absorption and secretion To circumvent the mucosal blood countercurrent exchanger tracers confined to the intravascular compartment were used For this purpose radioactively labelled blood cells and plasma particles were introduced into the circulation

a Experimental procedures
The technique involves a slug injection of a known amount of blood or plasma labelled with the β-emitting tracers ^{32}P or ^{198}Au administered into the superior mesenteric artery and detected by means of sensing devices placed in the gut lumen in close approximation to the villi Thus the intravascular transit of the tracer particles were registered by means of cylindrical detectors with their sensitive surface in close mucosal contact Since the tissue volume monitored by the detector depends on the energy level of the β-radiation the ^{32}P-labelled red cells and plasma colloids were monitored largely from the entire mucosa while ^{198}Au-labelled plasma particles were registered essentially only from the villi (I) The total blood supply to the intestinal segment studied was estimated from a continuous registration of the venous outflow

The appearance of the tracer in the mucosal vascular compart-

ments after a slug injection was recorded as an indicator-dilution curve which was then used for calculating mucosal blood circulation parameters The possibilities of measuring regional blood flow blood volume and mean transit time from these curves will be discussed below It should be pointed out that what is actually measured in the present series of experiments (I-IV) are the flow regional volume and mean transit time for red cells when using them as tracer vehicles and the same parameters for plasma when using colloid tracer particles

b Estimation of regional blood flow
When the amount of tracer and the blood flow in the injection artery are known the blood flow in the tissue region monitored by the detector can be estimated from the maximal height of the recorded indicator-dilution curve provided certain conditions are met (paper I) These conditions include even distribution of the injected tracer within the monitored tissue volume when the curve reaches its maximal height If this is the case the measured tracer concentration will be proportional to the blood flow in that region When using ^{198}Au for determination of villous blood flow there are good reasons to believe that this assumption is justified since the villous capillaries are arranged as a dense plexus Also when using ^{32}P for determinations of mucosal blood flow the curve maximum probably represents a situation where the tracer particles are fairly evenly distributed within capillaries since most of the afferent and efferent vessels are in fact more deeply situated and therefore generally speaking recorded with a lower efficiency than the capillaries However the intravascular compartment is probably unevenly distributed within the monitored volume in the ^{32}P experiments (I) Attempts to verify experimentally these inherent assumptions of the present technique were presented in paper I to which the reader is referred for details

c Estimation of regional blood volume
1 Perfused volume If the amount of injected tracer and the flow in the injection artery are known the blood volume in the monitored tissue region can be estimated from the area under the curve under certain conditions (paper I) These conditions include an even distribution of tracer in the intravascular volume being monitored and also good mixing between tracer and blood

If the injected bolus is incompletely mixed with the blood this will mean that the population of pathways used by the tracer particles will not be representative for the blood flow distribution However if

the mixing is incomplete in a randomized way such an error will be largely eliminated when repeated injections are performed (cf the theoretical situation of repeated injections of only one tracer particle) It was shown experimentally (I) that a fairly good mixing occurred between tracer and blood and since repeated injections were made there are reasons to believe that the regional blood and plasma volumes are accurately measured both when using ^{198}Au- and ^{32}P-labelled blood When ^{198}Au is used a recirculation of blood (and tracer) particles in a mesh of the capillary network of the villi may however add to the measured volume which is not the case when the volume is estimated as described below (see 2) Further the plasma or red cell volume measured with the slug injection technique includes only those vessels which were open for perfusion at the time for injection It was therefore named perfused regional red cell or plasma volume

2 "Total equilibrated volume Another way to estimate the regional blood volume is to let tracer particles equilibrate in the circulating blood and then compare the tracer concentration in the blood with that of the tissue If the time for equilibration is sufficiently long the volume thus estimated will also include the content of vessels that are intermittenly closed and was therefore named total equilibrated volume This technique could only be used for labelled red cells since the colloid particles if present in the blood for longer periods are in part trapped by reticulo-endothelial cells in the monitored tissue

d Estimation of mean transit time and linear flow rate
From the known relationship between flow volume and mean transit time it is evident that the mean transit time in this case can be calculated as the area of the indicator-dilution curve divided by its maximal height This value is not dependent of a perfect mixing between tracer and blood When using ^{198}Au-labelled plasma particles, their passage along the central arterial vessel of the villi adds very little to the transit time since the blood volume of the subepithelial capillary network is some 10 times larger than that of the central artery Knowing the height of the villi the average flow rate in the villous capillaries can be calculated If some rec rculation occurs in the mesh of the villous capillary network this will influence the curve area but probably not the curve height and will therefore be measured as a slower transit Thus it is mainly the linear flow rate from villous tip to base that will be estimated

Using ^{32}P as the tracer the transit time in capillaries will probably dominate but transit in venous vessels will also to a certain extent influence the curve

Results

The mucosal blood circulation of the small intestine was studied in animals deprived of food for 24 h and after acute intestinal denervation during resting conditions (II) during hyperemia (II) during reduced perfusion pressure (III) and during direct stimulation of the regional sympathetic nerves (IV)

Regional flow and volume are expressed per unit regional tissue weight, below and in Table 1 Mucosal weight constitutes 45 per cent of the total intestinal weight (Jodal and Lundgren 1970 b) and villi about 15 per cent as estimated in the present studies (I) Villous blood flow was calculated from the plasma flow passing through the villi as monitored by the ^{198}Au – labelled plasma particles (I) All parameters measured with this tracer are therefor designed villous On the other hand ^{32}P – labelled blood particles were seen by the detector largely throughout the mucosa and all parameters estimated with this tracer are therefor denoted mucosal

a Volume flow of blood

The total blood flow of the intestinal segment with its mesentery and lymph nodes was 15-30 ml/min x 100 g during "resting conditions a value somewhat lower than that earlier reported for the fasting cat (Lundgren 1967) This discrepancy is probably due to the fact that shorter intestinal segments were used in the present experiments implying that lymph nodes with their somewhat lower blood flow constituted a higher weight fraction

The mucosal blood flow was 20-25 ml/min x 100 g mucosa at rest i e it almost equals the total blood flow per unit weight intestinal tissue The "villous plasma flow during resting conditions varied between 10 and 25 ml/min x 100 g a comparatively large variation possibly explained by the villous arterioles being highly sensitive to metabolic factors (see below) Assuming a villous hematocrit of only 60 per cent of the arterial one (Jodal and Lundgren 1970a) and a similar linear flow rate for red cells and plasma in the villous capillary network the villous blood flow can be calculated to 15-30 ml/min x 100 g villous tissue Since the weight of the villi amounts to about 15 per cent of the total intestinal weight 10-20 per cent of the "resting" intestinal blood flow was distributed to the villi

During close i a infusions of isopropylnoradrenaline (II) the

total intestinal blood flow was increased maximally to about 200 ml/min x 100 g The mucosal blood flow during the same conditions amounted to 175-200 ml/min x 100 g The "villous plasma flow increased relatively more than the total intestinal blood flow corresponding to a "villous blood flow of 300-400 ml/min x 100 g which in this situation constitutes 30-35 per cent of the total intestinal blood flow

During graded reductions of the arterial inflow pressure (III) the total intestinal blood flow was reduced relatively less than the perfusion pressure illustrating the wellknown autoregulation of intestinal blood flow (e g Johnson 1964) During these conditions the "mucosal blood flow behaved like the total intestinal blood flow while the "villous blood flow exhibited the most pronounced autoregulation remaining almost unaltered down to pressure levels around 30 mm Hg As arterial inflow pressure was lowered an increasing fraction of the intestinal blood supply was therefore distributed to the villi increasing from 10-20 to 30-35 per cent at the lowest perfusion pressures

As venous outflow pressure was raised the total intestinal blood flow was reduced out of proportion to the perfusion pressure decrease due to a rise of the regional flow resistance This response ascribed to a constriction of arteriolar vessels has repeatedly been described and named the venous-arteriolar response (see Johnson 1964) When venous outflow pressure was raised from 10 to about 25 mm Hg "mucosal as well as "villous blood flow seemed to be reduced somewhat less than the total intestinal blood flow

Stimulation of the regional sympathetic nerves induced a characteristic change of the total intestinal blood flow (Folkow et al 1964 Wallentin 1966; for further ref see Shanbour and Jacobson 1971) First a strong but transient reduction occurs which however partly disappears after 1-2 min despite continued stimulation (autoregulatory escape from vasoconstrictor fibre influence) after which flow stabilized somewhat below control (steady state phase) When stimulation is stopped a transient hyperemia exceeding prestimulatory control occurs

Both mucosal and villous flow showed an initial strong reduction upon vasoconstrictor fibre stimulation During the steady state phase the "mucosal blood flow had returned towards control while the "villous blood flow exceeded control in face of a reduced overall intestinal flow Thus a considerable redistribution of the intestinal blood flow had evidently occurred during the steady state phase villous blood flow being increased while flow in some deeper part probably around the crypts had decreased This becomes clear

if one subtracts villous blood flow from that of the total mucosa in the steady state phase of neurogenic vasoconstriction and is also supported by the observation that the "mucosal" but not the "villous vessels exhibited a poststimulatory hyperemia

b Blood content
The "resting blood content in the mucosa as estimated from the total red cell content and the hematocrit in the perfused mucosal vessels amounted to 4 0–4 5 ml/100 g but only 2 5–3 5 ml/100 g of this volume was constituted by perfused vessels in this situation The plasma volume within the perfused "villous vessels amounted to 1 0–2 0 ml or 1 5–3 0 ml of blood per 100 g villous tissue During intense hyperemia induced by isopropylnoradrenaline the mucosal blood content increased to 5 0–6 0 ml/100 g and the "perfused mucosal to at least 4 5–5 0 ml/100 g Concomitantly the "villous capillaries increased their plasma content to 3 5–4 0 ml or to 4 5–5 0 ml of blood per 100 g villous tissue It can be calculated that this would correspond to a situation where capillaries cover roughly one third of the inner surface of the villous epithelial cells

During reductions of the arterial inflow pressure mucosal blood content increased slightly and a larger portion of the mucosa was now steadily perfused Thus at an arterial pressure of 30 mm Hg the perfused blood volume amounted to about 4 0 ml/100 g mucosa while the volume of perfused "villous capillaries was almost doubled compared to rest i e 3 5–4 5 ml of blood per 100 g villous tissue

When raising venous outflow pressure to 25 mm Hg a prominent increase of mucosal red cell content was seen probably reflecting distended veins in deeper parts The volume of perfused mucosal vessels increased to only a small extent and that of perfused "villous capillaries was hardly affected

During sympathetic stimulation both the total and perfused mucosal blood volumes were initially reduced but increased during the steady state phase the perfused mucosal blood volume being then 3 5–4 0 ml/100 g i e distinctly larger than resting control Concomitantly the blood volume within the perfused villous capillaries was increased roughly 50 per cent

c Linear flow rates
The average linear flow rates can be estimated from the mean transit time ($t_{A/h}$) if the length of the vascular pathways is known The average linear rate of plasma flow in the villous capillaries was calculated in absolute figures assuming a villous length of 0 7 mm

TABLE I

	BLOOD FLOW ml/min x 100 g			Fraction blood flow to villi per cent	BLOOD VOLUME ml/100 g			Linear flow rate in villi mm/s
	Total intestinal*	Mucosal	*Villous		Total *mucosal	Perfused *mucosal	Perfused *villous	
RESTING BLOOD FLOW	15–30	20–25	15–30	10–20	4 0–4 5	2 5–3 5	1 5–3 0	0 10–0 15
HYPEREMIA (isopropylnoradrenaline)	200	175–200	300–400	30–35	5 0–6 0	4 5–5 0	4 5–5 0	0 70
REDUCED ARTERIAL IN FLOW PRESSURE ([illegible] mm Hg)	7–8	8–10	11 17	30–35	4 5–5 0	3 5–4 5	3 5–4 5	0 03–0 05
RAISED VENOUS OUT FLOW PRESSURE ([illegible] mm Hg)	10–15	14 16	10–15	13 17	4 5–5 5	3 5–4 0	1 5–3 0	0 06–0 08
SYMPATHETIC STIMULATION (8 Hz)	[illegible]	20–25	23–27	17 27	4 0–4 5	3 5–4 0	2 0–3 3	0 15–0 20

while that of the entire mucosa was determined only in relative terms

During rest the average linear rate of "villous plasma flow amounted to 0 10-0 15 mm/sec at a volume flow of blood corresponding to 15-30 ml/min x 100 g villous tissue The linear flow rate increased to 0 70-0 80 mm/sec during intense hyperemia in which situation the villous blood flow was some 3-400 ml and total intestinal blood flow some 200 ml/min x 100 g Concomitantly the red cell and plasma linear flow rates in the mucosa were increased about five times Red cell transit was faster than that of plasma probably reflecting the axial streaming of blood cells During arterial pressure reduction these mean transit times were reduced to 1/3 - 1/4 of control at pressures around 30 mm Hg while the average linear rates of villous plasma flow was then only 0 03-0 05 mm/sec Upon increases of venous outflow pressure red cell and plasma flow rates in *the mucosa were considerably reduced while that of the villi was less* affected

During the initial phase of vasoconstrictor fibre stimulation linear flow rates were markedly reduced in both the mucosa and in the villous capillaries while neither of them differed significantly from control in the steady state phase

Discussion

Mucosal blood circulation in the small intestine has been studied by several authors using different "tracer accumulation techniques (for ref see paper I and II) The present studies were performed with a new technique allowing a differentiation of the circulation in the intestinal villi as compared with that of the entire mucosa thus including both villi and the tissue surrounding the crypts The use of this method is however restricted to studies on animals in which intestinal motility is largely eliminated implying that the influence of motility on mucosal hemodynamics cannot be studied in this way However intestinal motility as induced by vagal stimulation at the "physiological range of frequencies hardly at all affects either total intestinal blood flow (Kewenter 1965) or intestinal capillary filtration coefficient (Lundgren personal communication)

Every vascular bed consists of a number of specialized series-coupled sections i e resistance vessels exchange vessels and capacitance vessels (see e g Mellander 1960 Folkow 1967) Plethysmographic and gravimetric techniques have made it possible to study reactions within these vascular sections also in the small intestine

(e g Johnson and Hansson 1962 Folkow et al 1963) However no attempts have then been made to study separately e g the mucosal vessels from this particular point of view The indicator-dilution technique makes it possible to follow separately the reactions of the mucosal resistance and capacitance vessels and also those affecting the exchange vessels and the results will be discussed along these concepts

The resistance function within the villous and mucosal circuits is reflected in the flow values obtained with ^{198}Au- and ^{32}P-labelled blood particles respectively When comparing the present mucosal flow values with those of earlier investigations the present ones are usually higher This difference is probably explained mainly by the partial exclusion of diffusible tracers from parts of the mucosa due to their shortcirculting in the intestinal countercurrent exchanger (Lundgren 1967 Kampp Lundgren and Sjöstrand 1968) a drawback not involved when intravascular tracers are used Thus Grim and Lindseth (1958) utilizing labelled microspheres, found that 50 per cent of injected spheres (diameter 20 µm) were trapped in the mucosa during resting blood flow conditions reflecting a blood flow distribution similar to that of the present studies where about 45 % of the flow was diverted to the mucosa

A comparison of the results obtained with the two tracers used suggests that the mucosal blood flow is inhomogenous the "villous blood flow being considerably higher during maximal dilatation than the average one of entire mucosal section Hence its deeper parts must contain a less well-perfused section However, it appears that another extremely well-vascularized area exists in deeper parts probably associated with cell renewal and secretion at the base of the crypts (Lundgren 1967)

The present study clearly indicates that the "villous resistance vessels exhibit an extraordinary high "resting tone since villous plasma flow could be increased almost 15 times (from 20 to 275 ml/min x 100 g) by vasodilator drugs implying maximal blood flow values around 400 ml/min x 100 g of villous tissue Total mucosal blood flow on the other hand could only be increased 8 times above resting level (from 25 to 180 ml/min x 100 g) The supplying arteries to the villi and to the remaining mucosal parts are identical up to the submucosal level Therefore the mentioned differentiated control of the "villous circulation must be localized to those sections of the ascending villous vessels which pass between the crypts since they lose their smooth muscle coat when entering the villi

Further villous blood vessels seem to be highly sensitive to

local metabolic factors This was clear from the pronounced autoregulatory capacity of the villous vessels following reductions in arterial pressure In fact lowering perfusion pressure from 100 to below 30 mm Hg did not significantly decrease villous plasma flow (III) This observation may in part be due to myogenic factors i e the modulating effects of transmural pressure on inherent vascular smooth muscle activity (Folkow 1964) Since, however an elevated venous pressure reduced villous blood flow in direct proportion to the reduced perfusion pressure without clear signs of any villous vasoconstrictor response it appears that metabolic factors are dominant in the control of villous blood supply

The dominance of local factors in villous blood flow regulation may also be corroborated by the regional response to sympathetic discharge Thus the initial resistance increase in the villous vascular bed was overridden within 1-2 min by antagonistic factors that may well be metabolic in origin

By comparing the villous vascular reactions with those of the entire mucosa the vascular reactions in deeper mucosal layers could be studied indirectly The resting (basal) vascular tone seemed to be less pronounced in these deeper resistance vessels than in the villous ones to judge from the effect of vasodilator durgs Further this vascular region showed a less pronounced autoregulation to pressure changes than the villous vessels and prolonged constrictor fibre stimulation caused a more sustained reduction of blood flow It is difficult to assess the response of the crypt circulation to an increased venous outflow pressure but there may well have been a certain reduction of blood flow Thus these vessels seem to be more affected by nervous influences than the villous vessels and may possibly also be more sensitive to transmural pressure changes

The capacitance function reflected in the measured blood content of the mucosa was estimated from the total red cell volume when using the equilibration method The volume values obtained by the slug injection technique reflected the capacitance changes only in the perfused vessels and varied probably more due to opening up or closure of capillary and small vein sections as regulated by e g arteriolar or sphincter activity

The mucosal blood content as calculated from total red cell volume and the regional hematocrit estimated with the slug injection technique (II) ranged between 4 and 5 ml/100 g mucosa during rest and increased to about 6 ml/100 g during hyperemia or when venous outflow pressure was raised from 10 to about 25 mm Hg Thus the maximal mucosal change of total ntestinal blood volume was only

about 1 ml/100 g which should be contrasted to the values obtained when studying an entire intestinal segment including its mesentery Thus Wallentin (1967) found that the resting blood content in the intestine and its mesentery could be either increased or decreased about 3 ml/100 g intestine The observed difference reflects the fact that a considerable part of the total intestinal blood volume is actually confined to the mesenteric veins

The precapillary sphincter function of the intestinal vascular bed was indirectly reflected in the present measurements of the perfused "villous plasma (blood) volume and also in the difference between perfused and total mucosal blood volumes (II) The basis for considering the perfused "villous plasma volume as an indirect measure of precapillary sphincter" activity is that the villous vascular bed consists almost exclusively of capillaries (cf Fig 1) implying that this plasma volume would estimate the number of perfused capillaries If so the present experiments suggest that only 30-40 per cent of the villous capillaries are simultaneously open to flow during "resting conditions while vasodilator drugs or a reduced arterial inflow pressure leads to an opening of almost all the capillaries to flow During the steady state phase of sympathetic stimulation the fraction of perfused villous capillaries exceeded control slightly while it was hardly affected by increases of venous pressure Thus the villous precapillary sphincters showed responses similar to the villous resistance vessels both being e g highly sensitive to metabolic factors (see above) It is in fact likely that the resistance and precapillary functions of the villous vascular bed are anatomically overlapping and localized in the deeper part of the ascending arterioles where a smooth muscle coat is present If so a sphincter closure is likely to close off temporarily almost the entire flow of blood to a given villus

Therefore the present results suggest that the perfused volume in the villous tissue is largely proportional to the number of perfused villi though it is possible that local rheological factors may also influence the flow within the villous capillary meshwork The findings during direct mucosal inspection upon slug injections of plasma coloured by Evans blue corroborates the hypothesis of the villus being the smallest "unit of blood flow regulation since well defined uncoloured spots were seen during rest but disappeared during maximal vasodilatation (I)

The precapillary sphincter activity within the mucosal circuit as a whole can be estimated from the difference between total and perfused red cell or plasma volume This volume difference is pro-

bably explained by the presence of vessels mainly capillaries, which are not perfused by blood at the time of the slug injection because of upstream closure of sphincter sections" The precapillary sphincter function of the entire mucosa showed largely similar reactions as was described for the villi Thus only about 50–60 per cent of the mucosal vascular bed appeared to be perfused during rest while all vessels could be opened up by e g vasodilator drugs Similarly when reducing the arterial blood pressure a compensatory sphincter relaxation was reflected as a reduced difference between total and perfused volumes Further a raised venous outflow pressure appeared to close some sphincters while sympathetic stimulation during the steady state phase did not markedly alter the sphincter activity

A possible functional arrangement of the mucosal vascular bed
According to classical studies on the mucosal vascular arrangement in the small intestine (see Morphological considerations) the capillary networks in the villi and around the crypts are supplied by separate arterial vessels from the level of the submucosa The arterial vessels to the villi are non-branching arterioles ascending to the villous tips losing their muscular coats already at the villous base The major part of the adrenergic nerve endings in the mucosa are situated in the deeper part of the crypt region Furthermore from the present studies it seems probable that the arterial vessels supplying the villi are highly sensitive to metabolic factors while those supplying the crypts may be more sensitive to myogenic factors (see above)

Based on these two observations it is proposed that the arterial vessels supplying the villi are to a large extent controlled by the metabolic environment in the crypt region i e far from the villous capillary network that is exposed to an environment widely varying in its composition due to the impact of the intestinal contents The chemical environment of the crypt region is probably dependent mainly upon the metabolic activity in the secretory cells and in the region where cell renewal occurs Assuming such an arrangement and assuming that the capillaries around the crypts are highly porous one may explain the observations reported earlier and in this study concerning the reactions of the consecutive vascular sections during various experimental conditions

Thus the autoregulatory escape from vasoconstrictor fibre influence would be explained as follows Initially both villous and crypt arteriolar vessels constr ct However the accumulation of local chemical factors in the tissues su rounding the crypts eventually overrides the nervous effect on the v llous arterioles while those running

to the crypts remain constricted thereby producing a local chemical environment that opens up the closely adjacent villous arterioles Thus blood flow is redistributed from the highly porous capillaries of the crypts proper to the villi explaining the reduced capillary filtration coefficient (CFC) observed during these conditions (Folkow et al 1964 a b)

An increase of the venous outflow pressure constricts predominantly the arteriolar vessels supplying the crypts due to their response to an increased distending pressure Metabolites then accumulating in the crypt tissues tend to antagonize a possible constriction of the villous arterioles that seem to be especially sensitive to metabolic influences Thus blood flow is redistributed from the highly porous crypt capillaries to the villi in a similar way as during neurogenic vasoconstriction A marked reduction of intestinal CFC upon venous pressure increases has also been reported by Johnson (1964)

Upon marked reductions in arterial pressure an accumulation of metabolites in the crypt region will dilate the villous arterial vessel and to such an extent that villous blood flow stays largely constant despite the lowered perfusion pressure Most "villous precapillary "sphincters" open up as reflected in the markedly increased perfused "villous plasma volume (III) Concomitantly intestinal CFC increased 50 per cent above control (Haglund and Lundgren 1972) If "villous" plasma volume is augmented to the same extent by i a infusions of isopropylnoradrenaline a 4-6-fold increase of CFC is observed (Folkow Lundgren and Wallentin 1963) According to the present hypothesis these observations are explained by the fact that the drug causes vasodilatation in all intestinal wall layers including the crypt vascular circuit with its highly porous capillaries while these are opened up to a far less extent during arterial hypotension

PASSIVE ABSORPTION IN THE SMALL INTESTINE DURING DIFFERENT BLOOD FLOW CONDITIONS IN THE MUCOSA

The relationship between total intestinal blood flow and rate of intestinal absorption has been studied by several authors Thus the absorption of basic nutrients like glucose and amino-acids was found to be dependent on intestinal blood flow though primarily because of its importance for the metabolism of the actively absorbing cells (for ref see paper V). The absorption of water as well as of a number of pharmacological substances and different gases has also been studied with respect to blood flow dependence However in all these studies changes of total intestinal blood flow were induced without knowing how exactly the experimental procedures affected the blood flow in the superficial mucosal layers where absorption takes place Since the villous tissue constitutes only about 15 per cent of the small intestinal intramural tissue weight it seems probable that large variations of blood flow may occur in this tissue compartment without being reflected as corresponding changes of the total intestinal blood flow This conclusion is corroborated by observations described in the preceding chapter clearly showing that the villous blood circulation does not necessarily vary in direct proportion to changes of total intestinal blood flow

Knowing the hemodynamic characteristics of the mucosal vascular bed from the presently described studies it was regarded of interest to study in the first hand the passive absorption from the intestinal lumen in an attempt to clarify the relationship between this absorption and the blood flow rate As regards this relationship Winne (1967 1971 b) has outlined a theory for the movement of substances from the lumen of the small intestine to the intestinal blood stream using theoretical models with two to four compartments However these theoretical models seem to be based on simplified conditions where e g the countercurrent exchange between ascending and descending vessels of the mucosa are not taken into account

In the present investigations absorption is measured as the appearance of a test substance in the venous blood draining the small intestine Thus the absorption is here discussed with reference to a system made up of two compartments (lumen and blood) though interposed by an unknown number of not defined compartments With respect to hemodynamic changes, countercurrent exchange plasma skimming etc the mucosa is in fact so complex as to hardly allow at present the construction of any detailed model for the passive absorption from the lumen

An easily diffusible lipid soluble gas ^{85}Kr was chosen as the

test substance since it is metabolically inert and largely excluded from the circulation after a single pulmonary passage, keeping the arterial concentration virtually at zero Furthermore, ^{85}Kr has been shown to be extravascularly shunted in the mucosal countercurrent exchanger after intra-arterial administration (see Lundgren 1967) a mechanism that has been proposed to delay net absorption of easily diffusible solutes

Methodological considerations

The primary interest in these studies was to investigate the correlation between mucosal blood circulation and rate of absorption In the experimental technique used attempts were therefore made to eliminate other factors of importance for absorption For this purpose saline containing ^{85}Kr an inert and rapidly diffusible radioactive isotope was perfused through the lumen of a gut segment at a rate high enough to largely eliminate intraluminal concentration differences The absorbed tracer amount was estimated from its appearance in the intestinal venous blood as continuously measured with a well type scintillation counter The recorded radioactivity gave a direct measure of the amount absorbed since the tracer concentration in the arterial blood was negligible, ^{85}Kr being largely excluded from the circulation after a single pulmonary passage Since the mesentery with its vessels was covered with Mylar® (Du Pont) this prevented significant diffusion losses of the tracer, as was also checked in control experiments

The absorption rate could then be calculated as the product of the venous outflow and its tracer concentration and could be directly compared to the tracer concentration in the luminal perfusate In analogy with the clearance concept in the kidney it is possible to calculate the amount of intraluminal fluid that per unit time is cleared of the tracer However since the primary interest was to relate the rate of absorption to the blood flow it appeared more useful to express the absorptive capacity in terms of the volume of intestinal blood that per unit time was fully equilibrated with the luminal contents Knowing the partition coefficient for ^{85}Kr between saline and blood the mentioned absorptive capacity could easily be estimated from the measured parameters

To study the distribution volume in the intestinal wall of an easily diffusible substance during absorption an autoradiographic study was performed using another tracer antipyrine-N-methyl-^{14}C ^{85}Kr was not suitable for this part of the study because of its comparatively high energy β-radiation and the difficulties inherent in avoiding its evaporation from thin tissue slices The localisation of the tracer in

the intestinal wall, as indicated by the blackness of the autoradiographs was determined by simultaneous microscopical examination of the histological sections and the corresponding autoradiographs

Results

The absorption of ^{85}Kr from the lumen of the small intestine was studied during resting blood flow during hyperemia (V) during reduced arterial inflow pressure during raised venous outflow pressure and during the influence of vasoconstrictor fibre activity (VI)

"Resting" blood circulation At a total intestinal blood flow of 25 ml/min x 100 g about 4-5 ml of blood per min and 100 g intestine was fully equilibrated with the luminal contents i e about 17 per cent of the intramural blood flow was fully equilibrated with the luminal ^{85}Kr concentration Autoradiographic studies showed that an easily diffusible lipid soluble substance when introduced from the lumen during the present experimental conditions becomes spread throughout the entire mucosal tissue layer which during rest is supplied with about 45 per cent of the total intramural blood flow while the concomitant part to the villi is about 15-20 per cent

Hyperemia During infusion of isopropylnoradrenaline in a dose that produces a moderate intestinal hyperemia about 100 ml/min x 100 g the amount of absorbed ^{85}Kr was increased to about 13 ml/min x 100 g while the fraction of intestinal blood flow that was fully equilibrated with the intestinal contents decreased to about 12 per cent Thus the absorption rate increased proportionally less than the intestinal blood flow Autoradiographic studies demonstrated that the distribution volume for an absorbed lipid soluble substance was during hyperemia confined only to the villi At these levels of total intestinal blood flow 30-35 per cent is distributed to the villous capillaries (II)

Reduced arterial inflow pressure When the arterial inflow pressure was reduced the absorption rate showed a reduction in proportion to the decreased total intestinal blood flow Thus at an arterial pressure of 25-30 mm Hg and a venous outflow of 5 ml/min x 100 g the absorption rate was only 0 9 ml/min x 100 g Concomitantly the fraction of intramural blood flow that was fully equilibrated with the luminal contents showed a slight reduction as compared to rest probably due to some tracer diffusion from the venous blood to the surrounding air The autoradiograph c experiments demonstrated a distribution volume which included the entire mucosa as during resting flow During this conditions the mucosal blood flow constitutes about 50 per cent and the villous blood flow about 30-35 per cent of total

Raised venous outflow pressure During increases of venous outflow pressure the absorption rate showed a similar relationship to total intestinal blood flow as that observed upon reduction of the arterial pressure Thus at a venous outflow pressure of about 25 mm Hg and a venous outflow of 14 ml/min x 100 g the absorption rate was about 2 2 ml/min x 100 g corresponding to a fraction of intramural blood flow fully equilibrated with the luminal contents about 15 per cent During these conditions the distribution volume included the entire mucosa The fraction of intramural blood flow that passes through the villous capillaries is then slightly increased compared to rest (III)

Stimulation of regional sympathetic nerves During the steady state phase of sympathetic nerve stimulation the absorption rate was similar to prestimulatory control despite a moderate reduction of venous outflow The fraction of the intraluminal blood flow fully equilibrated with the luminal contents increased during the same conditions to about 22 per cent The autoradiographic studies showed that the tracer was distributed within the entire mucosa The fraction of intramural blood flow that passes through the entire mucosa then shows a slight increase while the fraction of flow diverted to the villous capillaries during nerve stimulation is considerably increased compared to rest (IV)

Discussion

In this series of experiments it was shown that the absorption rate of ^{85}Kr from the small intestine could be markedly changed by altering its total blood supply However the absorption rate was during resting" blood flow conditions lower than what could be expected from the concomitant mucosal blood supply Intestinal hyperemia as induced by isopropylnoradrenaline increased the absorption rate compared to control while a raised venous outflow pressure or a reduced arterial inflow pressure decreased the rate of absorption Increased activity in the regional sympathetic nerves on the other hand did not markedly affect the absorption rate despite a concomitant reduction of total intestinal blood flow

Generally speaking earlier reports in the literature concerning the absorption of easily diffusible solutes are in agreement with the present findings Grim Lee and Visscher (1955) reported that only 5 per cent of the small intestinal blood flow become fully equilibrated with D_2O placed in the lumen of the gut in a dog The concomitant

tracer concentration in the mucosa was 25 per cent of the luminal one Thus if about half of total intestinal blood flow is distributed to the mucosa the tracer concentration in the venous blood would be expected to be 10–15 per cent Furthermore Winne (1971 a) studied in rats the effects of changed intestinal blood flow on the rate of absorption for a variety of substances When increasing the intestinal blood flow an increased absorption rate was seen for lipid soluble solutes (e g antipyrine methanol) and for small molecular unionized water soluble solutes (e g tritiated water) while absorption rate of water soluble solutes of larger molecular weight (e g urea erythrit) was largely unaffected by blood flow

Intestinal absorption of gas with regard to intestinal blood flow has been studied by several authors Thus Schoen (1925) considered the blood flow as an important factor in elimination of gas from the small intestinal lumen of the dog since the absorption rate for several gases studied was found to be proportional to their solubility in blood Further Monroe et al (1926) showed that the intestinal absorption of gaseous O_2 CO_2 and H_2 was retarded during induced reductions of the intestinal blood flow which also was recently demonstrated for gaseous CO_2 by Pals and Steggerda (1966) In a study by Coburn (1968) the elimination of gaseous carbon monooxide from the intestinal lumen of the rabbit was shown to be highly dependant on blood flow while Hamilton Dawson and Webb (1967) failed to find any correlation between the absorption of ^{133}Xe dissolved in saline, and intestinal blood flow Further the concentration of ^{133}Xe in intestinal venous blood was only 2–5 per cent compared to the luminal fluid but the intestinal segment was not continuously perfused with the ^{133}Xe solution which to some extent may explain the findings Thus it is evident from earlier studies that intestinal blood circulation is an important factor for the elimination rate of gas in the intestinal lumen

The passive absorption of different substances by the small intestine may be limited by diffusion by blood circulation or by a combination of these factors The former type of limitation implies that the absorption rate is determined by the properties of the test substance with respect to its diffusion in the tissues while in the latter case the absorption rate is determined mainly by the mucosal blood flow and its arrangement as well as by the solubility of the test substance in blood (see Forster 1967) The results of the present studies will be discussed along these lines A comparison between the absorption rate and the rate of mucosal blood flow during the present experimental cond tions and mucosal hemodynamic situations may render it possible to elucidate which variables that are the most important ones for absorption

Absorption rate and blood flow As regards the blood flow of the entire intestinal wall it is evident that this flow cannot be the only factor determining the absorption rate Thus the intramural blood flow could be reduced by activation of the sympathetic vasoconstrictor nerves without any concomitant reduction of the absorption rate

When comparing the absorption rate with the mucosal blood flow it is evident that the absorption rate during e g "resting conditions was lower than what could be expected if the luminal contents were equilibrated with the entire mucosa which in fact was suggested by the autoradiographic studies It might however be argued that a concentration gradient existed between lumen and capillary blood in all or at least in the more deeply situated mucosal capillaries and that a combination of flow and diffusion limitations in this way was at hand If this were so it would be possible to increase the tracer concentration in the venous blood draining the mucosa by reducing mucosal blood flow However decreasing the mucosal blood flow whether by reducing arterial inflow pressure or by raising venous outflow pressure did not increase the mucosal venous tracer concentration (see Fig 2)

Finally the villous blood flow might be the rate limiting factor for the absorption In fact the villous blood flow and the absorption rate when expressed as ml of blood that is fully equilibrated lumen per time unit agreed well during resting conditions However the autoradiographic findings of a distribution volume that included also deeper mucosal parts during resting conditions argue against villous flow being the sole rate limiting factor Further, it is evident from Fig 2 that there was a great divergence between villous blood flow and absorption rate during moderate hyperemia A possible diffusion limitation across the intestinal epithelium was ruled out by calculations based on physical and morphological data (paper V) which show that the tissue sheet interposed between lumen and villous capillary blood will not constitute any significant diffusion barrier for ^{85}Kr Further the findings of an increased divergence between villous blood flow and absorption rate during a reduced villous blood flow cannot be explained by a diffusion limitation

To conclude it seems difficult to ascribe the regulation of the intestinal absorption rate for an easily diffusible lipid soluble substance to changes in either total intestinal blood flow mucosal blood flow or in villous blood flow when taken alone A combination of diffusion and flow limitations is also ruled out as discussed above

Absorption rate and capillary surface area If blood flow to some area of the intestinal mucosa is temporarily stopped by e g pre

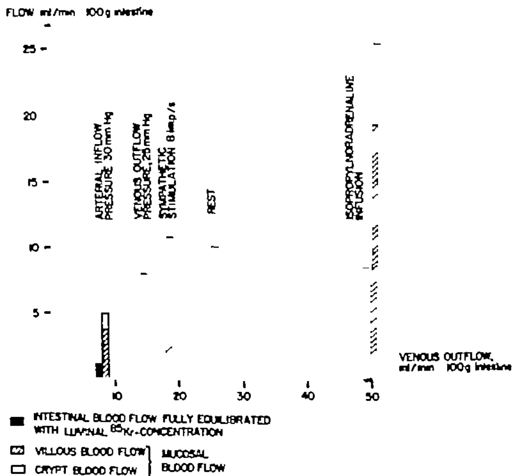


Fig 2 Approximate values on villous blood flow crypt blood flow and amount of blood that is fully equilibrated with the lumen during different experimental conditions in the small intestinal vascular bed All parameters are expressed in ml/min x 100 g intestinal tissue Crypt blood flow was calculated by subtracting villous flow from the mucosal flow

capillary sphincter activity it could be suspected that this would influence the absorption rate

From the hemodynamic studies it seems evident that a high sphincter activity doe occur in the mucosal vascular bed during conditions of rest a ~d venous outflow and sympathetic stimulation During reductions of th art rial inflow pressure and during hyperemia there seems to be a more e an perfusion of largely all the capillaries

Further the precapillary sphincter activity in the vascular bed of the villus and the crypt tissues seem to be changed largely in parallel during the studied conditions with the possible exception of sympathetic stimulation

Comparing the conditions with a low sphincter activity (hyperemia and arterial inflow pressure reduction) it seems evident that the perfused capillary surface area cannot be the dominating factor for the regulation of the absorption rate at least not for easily diffusible lipid soluble substances since the absorption rate widely differs during these conditions This also holds true when the absorption rate is related to the mucosal blood flow Thus, the blood leaving the mucosa during moderate hyperemia contains about 30 per cent but during a reduced arterial inflow pressure only 15-20 per cent of the luminal tracer concentration (see Fig 2)

Comparing conditions with a high level of precapillary sphincter activity the absorption rate may also markedly differ as when resting conditions are compared to the situation during raised venous outflow pressure In the latter situation the absorption rate is considerably below that during rest both in absolute figures and when related to the respective levels of mucosal blood flow Thus at "rest the blood leaving mucosa contains about 45 per cent of the luminal tracer concentration but during a raised venous outflow pressure only 30 per cent

Thus during conditions when roughly the same precapillary sphincter activity prevails in the mucosal vascular bed the absorption rate may differ widely and this also when related to the prevailing levels of mucosal blood flow These results indicate that the size of the available capillary surface area as regulated by the precapillary sphincter sections is not of paramount importance for the rate of absorption of easily diffusible lipid soluble substances

Absorption rate and linear flow rate for blood in the mucosal vessels
From the measurements of mean transit time $t_{A/H}$ it is possible to estimate in relative terms the average linear flow rate for red cells and plasma particles from $1/t_{A/H}$ In Fig 3 the absorption rate is plotted versus the average linear flow rates observed during the various circulatory conditions studied in the present series of experiments The illustrated values represent mean values taken from papers II-VI It is evident that there is a high correlation between absorption rate for ^{85}Kr and the linear flow rates for blood particles, both in villi and in the entire mucosa Thus there is e g a strongly reduced absorption rate during low arterial inflow pressure (VI) despite the fact that villous blood flow then stayed almost constant compared to rest and the number of perfused villous capillaries was increased However the

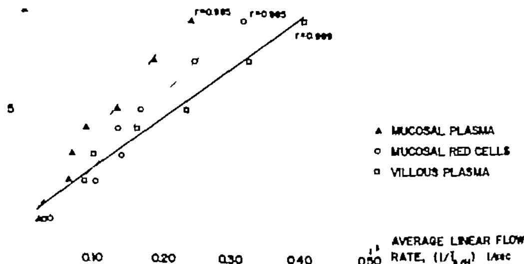


Fig 3 Absorption rate of ^{85}Kr (expressed in ml of blood that per min is fully equilibrated with the luminal tracer concentration) plotted versus average linear flow rates for red cells and plasma in mucosal and villous vessels (expressed as $1/t_{A/H}$) during the different experimental condition of the present study The range of linear flow rates corresponds to a total intestinal blood flow between 5 and 50 ml/min x 100 g The illustrated points represent observations made during rest hyperemia induced by isopropylnoradrenaline (2 levels) reduced arterial inflow pressure (2 levels) raised venous outflow pressure and sympathetic stimulation Lines constructed by the method of least squares r = correlation coefficient

linear flow rate in these capillaries and in the mucosal vessels was strongly reduced thus showing the important influence of the linear flow rate on the absorption rate

These findings concerning a close correlation between rate of absorption and linear flow rate for blood in the mucosal vascular hairpin loops agree well with the proposed function of the villous-mucosal countercurrent exchanger in the small intestine (see Lundgren 1967) as will further be discuss d below

The countercurrent exchange in the small intestinal mucosa as a factor limiting absorption of easily diffusible substances

The present findings of a low ratio between mucosal blood flow and intestinal absorption rate for an easily diffusible test substance suggest that other factors than the volume flow of blood limit the absorption This limitation cannot however be ascribed to a diffusion barrier between lumen and blood whether alone or in combination with a blood flow limitation (see above) It is proposed that the present findings of a comparatively low absorption rate of ^{85}Kr is explained mainly by the existence of a countercurrent diffusion exchange between ascending and descending vessels in the villi and adjacent parts of the mucosa delaying net intestinal absorption (Lundgren 1967)

According to this hypothesis the absorbed solute diffuses along a concentration gradient from the subepithelial capillary network to the central arteriolar vessel of the villus b ing thus again brought towards the villous tip In such a way easily diffusible solutes are delayed in entering the venous effluent by being "trapped in the mucosal countercurrent exchanger

The countercurrent hypothesis is corroborated by the high correlation observed between absorption rate and linear flow rate of blood in the mucosal hairpin vascular loops since the time available for exchange diffusion is determined by the linear rate of flow and hence by the mean transit time Thus it was demonstrated that the countercurrent exchange of arterially injected blood borne substances became insignificant when intestinal blood flow was sufficiently increased e g for urea at a total intestinal blood flow of 100 ml/min x 100 g and for ^{85}Kr and antipyrin at 150 ml/min x 100 g (Lundgren 1967)

The results of the present studies made it possible to compare the actual absorption rate with that present in case the mucosal blood flow had become saturated with the tracer If a difference is observed and it cannot be ascribed to diffusion obstacles it is believed to be largely due to the countercurrent exchange mechanism If so the efficiency of this mechanism can be estimated from the size of ratio between the expected and the measured absorption rate Such a comparison is however complicated by the fact that uptake of tracer seems to take place also in deep mucosal vessels during a low mucosal blood flow while it seems to be largely confined to the villous capillaries during intense hyperemia (V) Thus it is a question of which part of the mucosal blood flow that should be considered as absorptive

However during a reduced arterial inflow pressure and during moderate hyperemia the villous blood flow constitutes the larger part

of the mucosal blood flow (see Fig 2) and a rough comparison can then be made In these two experimental situations no diffusion limitation will exist between lumen and villous capillary blood (V) It can be calculated from Fig 2 that the ratio between expected and actual absorption rates is 3–4 5 at an arterial inflow pressure of 30 mm Hg the range of the observed values being due to which flow value that is chosen (villous or mucosal) The corresponding values during moderate hyperemia are 2 2–3 0 There are reasons to believe that almost the entire mucosal blood flow becomes equilibrated with luminal contents during a low flow state (VI) and thus the ratio would be more close to 4 5 than to 3 1 under those circumstances During hyperemia on the other hand the intraluminar tracer appears to be mainly distributed to the villi and the discussed ratio would then be closer to 2 2 than to 3 0 Thus an increase of intestinal blood flow and linear rate of flow in the mucosa reduces the difference between expected and actual absorption rate probably reflecting a decreased efficiency" of the mucosal countercurrent exchanger

The discussion above suggests that the hindering effect on absorption of the mucosal countercurrent exchange becomes decreased when blood flow is increased One may then pose the question if it is possible to increase intestinal blood flow to such an extent that virtually no exchange diffusion of ^{85}Kr can occur any longer Fig 3 showing the linear relationship between absorption rate and linear rate of flow makes it possible to answer this question if it is assumed that the linear correlation of Fig 3 can be extrapolated to higher blood flows If so such an extrapolation shows that within a physiological range of flow the absorption rate of ^{85}Kr will not be able to reach the values expected from the villous blood flow observed at maximal intestinal vasodilatation Since no diffusion limitation could exist between lumen and villous blood in the range where the extrapolation was made it may be concluded that the countercurrent exchange of absorbed ^{85}Kr cannot be entirely eliminated even at intense intestinal hyperemia This should be contrasted to the findings that the countercurrent exchange for blood borne ^{85}Kr and oxygen became fairly insignificant already at intestinal blood flows above 150 ml/min x 100 g This difference in countercurrent efficiency can be explained by the vascular arrangement in the villi with a high linear flow rate in the ascending arterial vessel and a slow one in the descending capillaries Such an arrangement will imply a higher efficiency for the countercurrent exchange when a test substance is introduced in the slower stream as was demonstrated in model experiments by Niesel and Röskenbleck (1963)

It is thus possible to imagine a situation where villous blood flow is increased to the extent that oxygen fully reaches the villous tips while there is still an impeding effect on the entrance of easily diffusible substances from the intestinal lumen to the intestinal venous blood

It should in this connection be underlined that the present technique for studying absorption was unphysiological in the sense that fluid containing ^{85}Kr was perfused through the lumen of the gut at a very high rate This experimental arrangement probably exposes a larger intestinal epithelial area to the intraluminal contents than would occur during more physiological conditions since it seems probable that only the upper parts of the villi are normally in efficient contact with the luminal contents The latter situation with absorption taking place mainly around the hairpin bends constitutes a situation with a high efficiency for the countercurrent exchange mechanism the more so since exchange diffusion of easily diffusible solutes like ^{85}Kr apparently occurs also in the deeper parts of the mucosa possibly even in the submucosa (cf Lundgren 1967)

SUMMARY AND CONCLUSIONS

1 A method is described for studying separately and quantitatively the blood circulation in the small intestinal mucosa of the cat The technique involves i a injections of β-radiating labelled blood particles the transit of which is detected in the intestinal tissue with β-detectors placed in the gut lumen Due to the energy level of that β-radiation ^{32}P-labelled blood particles were monitored from the entire mucosa while ^{198}Au-labelled plasma colloid particles were registered only from the villi From the recorded indicator-dilution curve and the measured total intestinal blood flow it was possible to estimate "mucosal or "villous blood flow blood volume and linear flow rate (I)

2 The following observations on the blood circulation in the small intestinal mucosa were made

a At resting levels of total intestinal blood flow (20–30 ml/min x 100 g of intestinal tissue) mucosal blood flow amounted to 20–25 ml/min x 100 g mucosal tissue and about 45 per cent of the intestinal blood flow was diverted to the mucosa "Villous blood flow was then 15–30 ml/min x 100 g villous tissue and 10–20 per cent of the intramural flow was distributed to the villi The blood content in the mucosal tissue was about 4 0–4 5 ml/100 g and the "villous blood volume amounted to 1 5–3 0 Only 50–60 per cent of the mucosal vessels and only 30 40 per cent of the villous capillaries were perfused during resting conditions The linear flow rate for blood in the "villous capillaries was estimated to 0 10–0 15 mm/s

b During intense vasodilatation (total intestinal blood flow around 200 ml/min x 100 g) induced by isopropylnoradrenaline the villous blood flow amounted to 300–400 ml/min x 100 g villous tissue and 30–35 per cent of intestinal blood flow was now diverted to the villi The corresponding values for "mucosal blood flow were 200 ml/min x 100 g mucosal tissue and 45 per cent The blood content of the mucosa amounted to 5 0–6 0 ml/100 g and in the villi to 4 5–5 0 ml/100 g Almost all vessels within the mucosa seemed to be perfused by blood The linear flow rate of blood in the villous capillaries now amounted to about 0 70 mm/s

c When reducing perfusion pressure by lowering arterial inflow pressure or raising venous outflow pressure villous blood flow was less affected than mucosal blood flow indicating that blood flow was reduced during these procedures in a deeper mucosal vascular compartment probably located around the crypts The villous blood flow exhibited an extremely high capacity for autoregulation and stayed

almost unchanged when arterial inflow pressure was reduced from 120-130 to 30-40 mm Hg At such a low arterial pressure most villous capillaries were open for perfusion then at a linear blood flow rate of only 0 05 mm/s

d During the steady state phase of sympathetic vasoconstrictor activation villous blood flow was slightly increased as compared to control while there seemed to be a consistent reduction of blood flow in the deeper part of mucosa The linear flow rates of blood in the mucosal vessels did not differ from resting control conditions

3 The results of the present study suggest that the blood vessels supplying the villi are highly sensitive to local chemical factors while nervous and myogenic factors probably dominate the resistance vessels supplying the deeper parts of the mucosa

4 A method was presented for studying passive abso n of an easily diffusable lipid soluble substance (^{85}Kr) from the smal testinal lumen during various circulatory conditions in the small intesti Absorption rate was estimated from the amount of tracer appearing in the mesenteric vein and expressed as volume of blood that per unit time was fully equilibrated with the luminal tracer concentration Intramural concentration gradients were eliminated by perfusing the gut lumen with a saline solution containing ^{85}Kr at a constant high rate

5 With the technique described above the following observations were made:

a During resting condition 15-20 per cent of intramural blood flow was found to be fully equilibrated with the luminal contents Absorption rate then amounted to 5 ml/min x 100 g intestinal tissue

b During a moderate intestinal hyperemia induced by isopropylnoradrenaline the absorption rate of ^{85}Kr increased and amounted to about 15 ml/min x 100 g intestinal tissue at a total venous outflow of 100 ml/min x 100 g intestine i e 15 per cent of the intestinal blood flow was fully equilibrated with the intestinal contents

c During a reduced perfusion pressure induced by lowering arterial inflow pressure or raising venous outflow pressure the absorption rate was reduced in proportion to the total intestinal blood flow

d During the steady state phase of sympathetic vasoconstrictor activation the absorption rate was not reduced despite a concomitant reduction of total intestinal blood flow

6 It was concluded from these observations that neither volume blood

flow nor diffusion constitute the rate limiting factor for the passive absorption of lipid soluble easily diffusible solutes Furthermore the villous capillary surface area was also found not to be a major determinant of the absorption rate of ^{85}Kr On the other hand a close correlation was observed between linear blood flow rate and rate of absorption of ^{85}Kr suggesting an important function of the countercurrent diffusion exchange between ascending and descending vessels in the mucosa of the small intestine

7 Some functional characteristics of the mucosal countercurrent exchanger with respect to the intestinal absorption of easily diffusible lipid soluble substances have been discussed

ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor in research, docent Ove Lundgren for his guidance co-operation and encouragement I am also very grateful to professor Björn Folkow for his interest support and valuable criticism throughout the work

My thanks are also due to docent Ingemar Wallentin who initially introduced me into this field of research

I also wish to thank my co-workers dr Björn Biber and civ ing Lars Stage for help and interesting discussions

For all skilful technical assistance I thank Mrs Dagny Johansson Mrs Lisbeth Lindgren and Mrs Eva Bengtsson For typing the manuscrips I thank Mrs Kerstin Lundgren and Mrs Sylvia Palmquist The valuable help of the animal keeper Gösta Magnusson and the photographer Thomas Palm is gratefully acknowledged as also the help of Elsa Fredriksson and Roger Eliasson

Thanks are also due to dr Roderick A Little for revising part of the English text in this summary

I also wish to express my gratitude to my wife Christina for her encouragement and support

This research has been sponsored by grants from the Swedish Medical Research Council (No B72-14X-2855) from the Faculty of Medicine, University of Göteborg and from Svenska Sällskapet för Medicinsk Forskning

REFERENCES

BAEZ S Microcirculation in the intramural vessels of the small intestine in the rat In The Microcirculation Urbana Ill Univ Illinois Press 1959 114-129

BIBER B O LUNDGREN and J SVANVIK An indicator-dilution technique for studying mean transit time and blood flow in the mucosa-submucosa of the small intestine Acta physiol scand 1969 Suppl 330

BOULTER P S and A G PARKS Submucosal vascular patterns of the alimentary tract and their significance Brit J Surg 1959 67 546-550

CASLEY-SMITH J R Endothelial fenestrae in intestinal villi: Differences between the arterial and venous ends of the capillaries Microvascular Res 1971 3 49-68

CLEMENTI F and G E PALADE Intestinal capillaries J Cell Biol 1969 41 33-58

COBURN R F Carbon monoxide uptake in the gut Ann Ny Acad Sci 1968 150 13-21

DELANEY J P Arteriovenous anastomotic blood flow in the mesenteric organs Amer J Physiol 1969 216 1556-1561

DRESEL P B FOLKOW and I WALLENTIN Rubidium86 clearance during neurogenic redistribution of intestinal blood flow Acta physiol scand 1966 67 173-184

FOLKOW B O LUNDGREN and I WALLENTIN Studies on the relationship between flow resistance capillary filtration coefficient and regional blood volume in the intestine of the cat Acta physiol scand 1963 57 270-283

FOLKOW B Description of the myogenic hypothesis Circulat Res 1964 14 Suppl 1 279-287

FOLKOW B D H LEWIS O LUNDGREN S MELLANDER and I WALLENTIN The effect of graded vasoconstrictor fibre stimulation on the intestinal resistance and capacitance vessels Acta physiol scand 1964a 61 445-457

FOLKOW B D H LEWIS O LUNDGREN S MELLANDER and I WALLENTIN The effect of the sympathetic vasoconstrictor fibres on the distribution of capillary blood flow in the intestine Acta physiol scand 1964b 61 458-466

FOLKOW B Regional adjustments of intestinal blood flow Gastroenterology 1967 52 423-432

FORSTER R E Physiological basis of gas exchange in the gut Ann Ny Acad Sci 1968 150 4-12

GRIM E J S LEE and M B VISSCHER, Water exchange between intestinal contents tissues and blood Amer J Physiol 1955 182 359-363
GRIM E and E O LINDSETH Distribution of blood flow to the tissues of the small intestine of the dog Minn Med 1958 30 138-145
HAGLUND U and O LUNDGREN Reactions within consecutive vascular sections of the small intestine of the cat during prolonged hypotension Acta physiol scand 1972 84 151-163
HAMILTON J D A M DAWSON and J WEBB Limitation of the use of inert gases in the measurement of small gut mucosal blood flow Gut 1967 8 509-521
HELLER A Uber die Blutgefässe des Dunndarmes Ber süchs Ges Wiss 1872 24 165-171
HORSTMANN E , Uber das Endothel der Zottenkappillaren im Dunndarm des Meerschweinchen und des Menschen Z Zellforsch 1966 72 364-369
JACOBOWITZ, D Histochemical studies of the autonomic innervation of the gut J Pharmacol exp Ther 1965 149 358-364
JACOBSON L F and R J NOER The vascular pattern of the intestinal villi in various laboratory animals and man Anat Rec 1952 114 85-101
JODAL M and O LUNDGREN Plasma skimming in the intestinal tract Acta physiol scand 1970a 80 50-60
JODAL M and O LUNDGREN Regional distribution of red cells plasma and blood volume in the intestinal wall of the cat Acta physiol scand 1970b 80 533 537
JOHNSON P C and K M HANSON Effect of arterial pressure on arterial and venous resistance of intestine J appl Physiol 1962 17 503-508
JOHNSON P C Origin localization and homeostatic significance of autoregulation in the intestine Circulat Res 1964 Suppl I 225-232
KAMPP M O LUNDGREN and J SJÖSTRAND The distribution of intravascularly administered lipid soluble and lipid insoluble substances in the mucosa and the submucosa of the small intestine of the cat Acta physiol scand 1968 72 469-480
KEWENTER J The vagal control of the jejunal and ileal motility and blood flow Acta physiol scand 1965 65 Suppl 251
LUNDGREN, O Studies on blood flow distribution and countercurrent exchange in the small intestine of the cat Acta physiol scand 1967 Suppl. 303

LUNDGREN, O and J SVANVIK Uptake of ^{85}Kr from the lumen of the small intestine to the intestinal blood in the cat Acta physiol scand 1968 74 20A 21A

MALL J P Die Blut- und Lymphwege im Dünndarm des Hundes Abh sächs Ges Wiss 1888 14 153-189

McIVER M A A C REDFIELD and E B BENEDICT Gaseous exchange betwen the blood and the lumen of the stomach and intestines Amer J Physiol 1926 76 92-111

MELLANDER S Comparative studies on the adrenergic neuro-hormonal control of resistance and capacitance blood vessels in the cat Acta physiol scand 1960 50 Suppl 176

MOHIUDDIN A Blood and lymph vessels in the jejunal villi of the white rat Anat Rec 1966 156 83-90

NIESEL W and H RÖSKENBLECK Die Bedeutung der Stromgeschwindigkeiten in den Gefässystemen der Niere und der Schwimmblase für die Aufrechterhaltung von Konzentrationsgradienten Pflügers Arch ges Physiol 1963 277 302-315

NISIOKA, T 1927 Cited by Spanner 1932

NOER J R The blood vessels of the jejunum and ileum: A comparative study of man and certain laboratory animals Amer J Anatomy 1943 73 293-325

NORBERG K -A Adrenergic innervation of the intestinal wall studied by fluorescence microscopy Int J Neuropharmacol 1964 3 379-382

PALS, D T and F R STEGGERDA Relation of intra-[illegible] carbon dioxide to intestinal blood flow Amer J Physiol [illegible] 893-896

REYNOLDS D G J BRIM and T W SHEEHY The [illegible] architecture of the small intestinal mucosa of the [illegible] mulatta) Anat Rec 1967 159 211 218

REYNOLDS D G and K G SWAN Intestinal [illegible] architecture in endotoxic shock Gastroenterology [illegible]

SCHOEN R Experimentelle Untersuchungen über Meteorismus [illegible] Teil Diffusion und Resorption der Darmgase [illegible] Bedingungen Dtsch Arch klin Med [illegible]

SHANBOUR L L and E D JACOBSON Autoregulatory escape in the gut Gastroenterology 1971 60 [illegible]

SILVA D G , G ROSS and L W OSBORNE Adrenergic innervation of the ileum of the cat Amer J Physiol [illegible]

SPALTEHOLTZ W Die Verteilung der Blutgefässe im Muskel Abhandl Sächs Gess Wiss Phys -Math [illegible] 14 [illegible] 528

SPANNER R Neue Befunde uber die Blutwege der Darmwand und ihre funktionelle Bedeutung Morph Jb 1932 69 394–454

WALLENTIN I Studies on intestinal circulation Acta physiol scand 1967 69 Suppl 279 1–38

WINNE D and H OCHSENFAHRT Die Formale Kinetik der Resorption unter Berucksichtigung der Darmdurchblutung J Theoret Biol 1967 14 293–315

WINNE D Durchblutung und enterale Resorption Z für Gastroenterologie 1971a 6 429 441

WINNE D Die Pharmakokinetik der Resorption bei Perfusion einer Darmschlinge mit variabler durchblutung Naunyn – Schmiedebergs Arch Pharmak 1971b 268 417–433

[illegible], Göteborg 1973

THE MECHANICS OF THE EXPIRATION

By

Ole Find Pedersen

From Marselisborg Hospital and Institute
University of Aarhus, Denmark

Arhus 1973

ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 386

From Marselisborg Hospital and Institute of Physiology

University of Aarhus Denmark

THE MECHANICS OF THE EXPIRATION EVALUATED BY A MODEL

By

Ole Find Pedersen

Århus 1973

This is a summary of a more extensive work dealing with the mechanics of the expiration:

Pedersen Ole Find:
The mechanics of the expiration evaluated by a model
Marselisborg Hospital - Institute of Physiology
Arhus 1973 (453 pages)

It may be obtained on reque t from the author as long as copies are available

References if not covered by the present summary will refer to thi more extensive work

Address Institute of Physiology
University of Århus
DK 8000 Århus C
Denmark

CHAPTER I

This chapter is an introduction to the present investigations with a discussion of the advantages and limitations of model theories The purpose of the present model experiments has been to describe the expiration by means of a new equation of motion taking into account the alinearity of the driving forces in relation to volume changing dimensions of the airways during the expiration and the effect of unequal ventilation of the different lung units The description includes the total airway as well as its different subdivisions as determined by the mechanical events during the forced expiration

CHAPTER II

The theoretical background has been evaluated systematically based on the equation of motion described by Mead (1961) The different terms of this equation have been evaluated one at a time starting with the elastic properties and further proceeding with the flow resi tive and inertial properties of th lungs

A major part of chapter II has been devoted to the theories dealing with the dynamic compression of the airways The term dynamic compression of the airways refer to the narrowing of the airway normally occurring during a forced expiration It i determined by the balance between the increased pleural pressure the

flow resistive pressure drops in the airways and the elasticity of the lungs and airways

The mechanical factors involved in the limitation of flow rate have been investigated The unique relation ship between pressure flow rate and volume of air in the lungs has been discussed in terms of the isovolume pressure-flow curves originally described by Fry Ebert Stead and Brown (1954) the Starling resistor concept of Pride Permutt Riley and Bromberger-Barnea (1967) and the equal pressure point (EPP) concept of Mead Turner Macklem and Little (1967) presenting different approaches in the description of the mechanics of the expiration

CHAPTER III

In this chapter a new equation of motion (actually consisting of three seperate equations) for the expiration has been derived (figure 1):

1) $P = K \dot{V}^n = k\ (\dot{V})^n$

P is the driving pressure V is the volume of air in the lungs beyond FRC or RV and $\dot{V}$ is the flow rate = $\frac{dV}{dt}$ which is negative due to the decreasing volume during the expiration K and n are constants relating the driving pressure to $\dot{V}$; k is the resistance when $-\dot{V}$ = 1 l sec^{-1} and n is an estimate of the degree of turbulence (n = 1 : laminar flow n = 1 75 : turbulent flow n = 2 0 flow through a constriction)

MODEL	RECTILINEAR LAMINAR	NOT NECESSARILY RECTILINEAR LAMINAR
DRIVING FORCE	$P \quad KV$	$P \quad KV^m$
RESISTANCE	$R \quad k \quad (P \quad k(\dot{V}))$	$R \quad k(\dot{V})^{n\ 1} \quad (P \quad k(-\dot{V})^n)$
	$KV \quad k(\dot{V}) \qquad \dot{V} \quad \frac{K}{k} V$	$KV^m \quad k(-\dot{V})^n \quad -\dot{V} \quad (\frac{K}{k})^{\frac{1}{n}} V^{\frac{m}{n}}$
FLOW VOLUME CURVES	$\dot{V}$; $\frac{K}{k}$; 1 ; V	$\log(-\dot{V})$; $\frac{m}{n}$; 1 ; $\log(\frac{K}{k})^{\frac{1}{n}}$; log V

FIGURE 1 The various components of th new equation of motion (right) compared with simpl model (l ft) in which the pressure is directly proportional to th airflow and to the volume

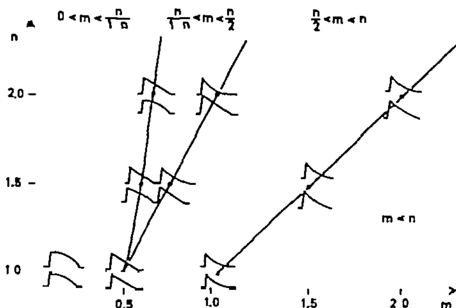


FIGURE 2 Corresponding pre sure curves (above) and flow curves (below) at different values f n and m

The logarithmic relationships between P V and -V are straight lines named the pressure-volume curve the pressure-flow curve and the flow-volume curve From the equations above other equations have been derived giving P V and -V as a function of time thus describing the pressure volume and flow curves Diagrams have been constructed showing curves for different values of m and n (figure 2)

As the equations above are valid only in case of airways with fixed dimensions it has been examined how changes in airway dimensions during the expiration influence the equations

Assuming a straight lined relationship between the conductance and the thoracic gas volume (Briscoe and DuBois 1958) it has been possible to show that with approximation

$$2) \quad k = k_3 V^s$$

where k_3 and s are constants and V as previously the volume of air in the lungs beyond FRC or RV Futhermore it has been shown that

$$3) \quad s = \text{FRC} \quad \frac{V}{Vtr + V}$$

where Vtr is the amount of trapped air in the lungs In this case V is the volume of air in the lungs beyond FRC but the equation may also be applied to the vital capacity by substituting RV for FRC

The equation $k = k_3 V^s$ expands the field of application of the new equation of motion and is called the function of normalization Substitution into the new equation of motion has resulted in a new set of normalized equations that are also straight lines on logarithmic scales These are determined by the constants m K n k_3 and s Not taking s into account in studies on human lungs will cause n (the degree of turbulence) to be underestimated

The effect of unequal ventilation has been examined from a theoretical point of view The degree of unequal ventilation may be expressed by the standard deviation (σ) of log-normal distribution functions of time constants ($= \frac{k}{K}$ when $m = n = 1$) Three 9 compartment systems have been examined ($\sigma = 0$ $\sigma = 0\ 27$ $\sigma = 0\ 89$) Two of these are shown in figure 3 Assuming that the volume of the overall system is the sum of the individual compartments that the flow rate is the sum of the individual flow rates and finally that the pressure is the volume weighted pressure average of the individual compartments it has been possible to calculate pressure-volume pressure-flow and flow-volume curves for the overall system Furthermore certain assumptions have been made regarding the initial pressures volume and functional residual capacities of the individual compartments These calculations how almost straight lined relationships (figures 4 5 6) They also show that the <u>overall driving pressure</u> expressed by m and K is influenced by the flow and accordingly is not the same during static and dynamic conditions (<u>cf</u> figure 4) In case of unequal ven

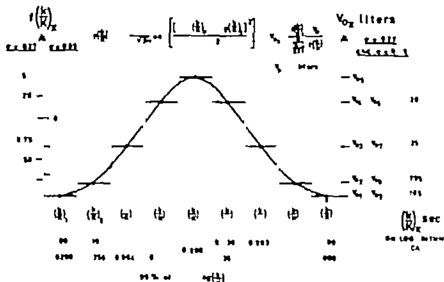


FIGURE 3 Two log-normal distribution fun tions f time onstants used in th evaluation f the effect of unequal ventilation on th new equation f motion

Δ log P

log V

FIGURE 4 Pressure-volume curve at two degrees of unequal ventilation Th slope m decreases with increasing unequality of ventilation (increasing σ) and the intercept log K increases

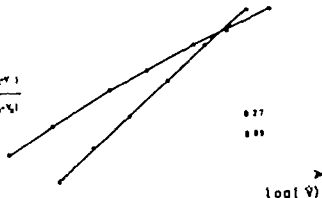


FIGURE 5 Pre sure-flow curves at two degrees of unequal ventilation The degree of turbulence measured by the lope n will be increasingly underestimated with increasing unequality f ventilation

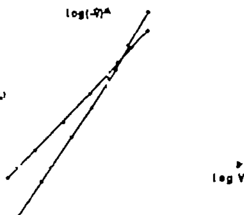


FIGURE 6 Flow-volume curves t two degrees f unequal ventilation Also in this ase the curves become flatte with increasing unequality of ventilation

tilation m decreases with increasing σ while K increases or decreases depending on the volume of the system It is furthermore concluded that the effect of unequal ventilation on the overall resistance is not different from the effect of static changes in airway dimensions Therefore the function of normalization has been applied to describe this condition too; s attains more negative values with increasing σ which is expected Finally it has been examined how the degree of frequency dependence of the dynamic compliance is related to the magnitude of s

The applicability of the new equation of motion to the peripheral airways has been evaluated in theory When used on the upstream segment of Mead et al (1967) the function of normalization is of value in the description of the motion of EPP If s is positive the upstream resi tance (measured by the constant k) decreases with decreasing V However both static changes in airway dimensions and unequal ventilation will tend to increase k during the expiration It is therefore most likely that this effect is overcompensated by an upstream motion of EPP when s is positive

When applied to the segment upstream from the site of compression of the airway according to Pride et al (1967) the function of normalization describes the changing dimensions of this segment in a similar way

The isovolume pressure-flow curves have been discussed in terms of the new equation of motion The contours of the irregular three dimensional surface

described by these curves are now straight lines on logarithmic scales when projected on the different planes The meaning of these lines are discussed with respect to the mechanics of the airways

CHAPTER IV

Chapter IV describes experiments with a mechanical model of the lungs

It has several subdivisions

Firstly the technical equipment is described The mechanical model (figure 7) is a closed transparent box containing two pairs of bellows with springs connected to the exterior of the box through connecting tubes for mounting of airways of varying collapsibility The equipment for measurement of pressures flow rates and volumes has been described and tested with respect to linearity and frequency response

Secondly the model experiments are described In the experiments with equal ventilation without change in airway dimensions only one lung has been used It is upplied with an incompressible airway Different sorts of resistances (stenoses and glasswool) and driving pres ures (bellows with springs and rubber bag) are used Related values of pressure flow volume and volume acceleration are recorded during passive expirations in order to demonstrate the different shapes of curves predicted from the equations It is found that the curves agree with the predictions and that the mechanical properties of the system are well

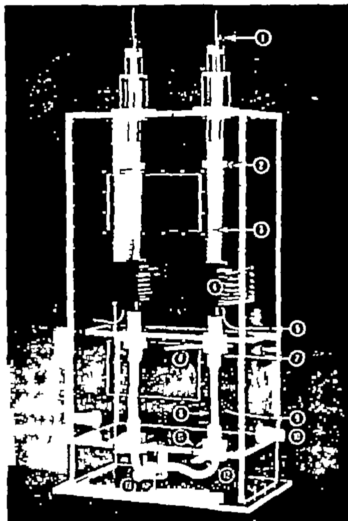


FIGURE 7

The mechanical model used in th experiments 1) and 2) screws f r adjustment f the aggregate with springs (3) 4) bellows 5) 7) 9) and 11) cannulas with tubings for measurement f the pressure inside the bellows the lateral airway pressure upstream from the collapsible segment the box pressure and the lateral airway pressure downstream from th collapsibl segment 6) and 12) slides with holes of various sizes to insert into the airway 8) collapsibl segment lo) connecting piece for external equipment 13) final common airway

defined by the constants m K n and k It has furthermore been demonstrated that the constants determining the pressure-flow curves can be calculated from the constants of the pressure-volume curves and the flow-volume curves This has been done in order to demonstrate that if two of the three relationships are known the third can be calculated It has finally been demonstrated that within given pressure intervals it is possible to convert the function $P = k(V)^n$ into $P = k_1(-V) + k_2(-V)^2$ so that the same relationship between P and V can be expressed by both functions with suitable values of n k k_1 and k_2

In the experiments with unequal ventilation without changes in airway dimensions both bellows have been applied, with springs adjusted so as to minimize the difference in driving pressure They are supplied with incompressible airways with different stenoses inserted to provide different resistance During the expiration they empty through the pneumotachograph back into the box containing the bellows in a plethysmographic set-up with a small spirometer to measure the volume changes of the box (figure 8) These experiments have shown that the dynamic pressures of each lung working separately are slightly lower than the static pressures probably due to resistance in the bellows (i e tissue resistance) Comparison of static and dynamic pressure-volume curves of both lungs working together doe not reveal any significant difference

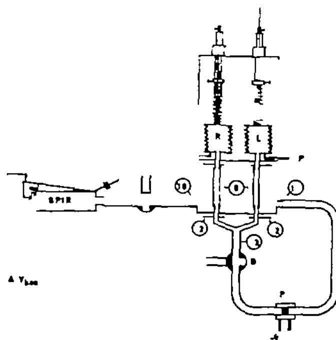


FIGURE 8 Diagram of the set-up us d in th experiments with unequal ventilation With the stopcock A open to the atmosph re the topcock B i turned o that air from a bottl with compressed air fills the bel lows The air supply is then topped without turning B leaving the bellows inflated but partly compres ed due to the tension of the springs A i next turn d in order to connect th box and the spiromet The expiration is started by sudden turn of B causing the bellows to empty through the pneumotachograph (PN) and back into th box At the end of th expiration the air in the bellows i no longer compressed and the volume f the box will be increased by th amount ΔV_{box} read on the spirometer The volume weighted pressure average an be al- culated from imultaneous values of ΔV_{box} volume contained in the bellows and barometri pre sure

although it is expected that m will decrease in dynamic as compared to static conditions in the presence of unequal ventilation The method however may not have been accurate enough for determination of small pressure changes On the other hand in accordance with the expectations it has been shown that n is underestimated in case of unequal ventilation Application of the function of normalization gives as expected a negative value of s

In the experiments with dynamic compression of the airways only one lung has been applied Four sets of experiments are designed in order to evaluate the effect of change in upstream resistance and airway collapsibility on the mechanics of expiration described by the new equation of motion Forced expirations are performed by increasing the box pres ure to varying preset levels The experimental set-up is shown in figure 9

Pres ure, flow, and volume curves have been obtained at different degrees of effort in each experiment and found to be in accordance with the concepts pre ented in th previous chapter

Isovolume p e sure-flow curves have been constructed in the four experiments Examples are shown in figures 10 and 11 They all show negative effort dependence i.e decreasing flow rate at increasing effort beyond a certain level; part from this they are in accordance with the predictions made From the study of these curves it seems relevant to use pressure flow

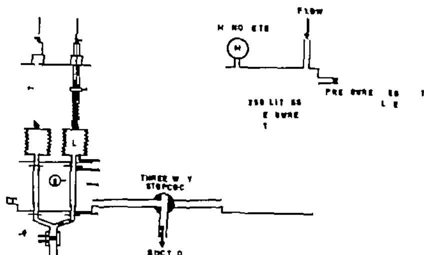


FIGURE 9 Diagram showing the mechanical model used in th experiment with forced expirations Only the left bellows (L) is used. The desired pressure in the pressure tank is btained by regulating th outflow resistance against a constant flow of air into the tank When suction is applied to the open end of the three-way stopcock the bellows fill A sudden turn of the stopcock to connect the pre sure tank and the box causes the pressure in the box to become positive to a degree depending on the initial tank pressure This again causes the bellows to expire PT pneumotachograph for measurement f xpired volume and flow P connections for measurement f pressure t different sites

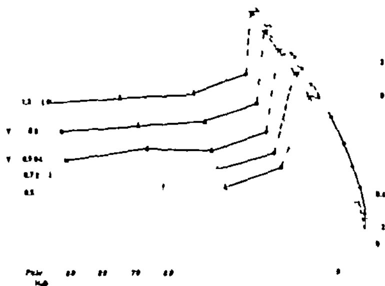


FIGURE 10 Isovolume pressure-flow curves from an experiment where the airway is abl to re ist a certain egative transmural pressure without collapsing Points with identical signatures are obtained from expirations with identical initial tank pressures (f legend to figure 9) Ascending unbroken curve pre sure-flow curve without connection f the pressure tank Dotted curve imilar curve but with added ext rnal stenosis As end ing broken curve (with start in the rigin) optimal pressure-flow curve determined by eparate experiment Horizontal bars on this curve are optimal point btained by interpolation on optimal flow-volume and pressure-volume curves

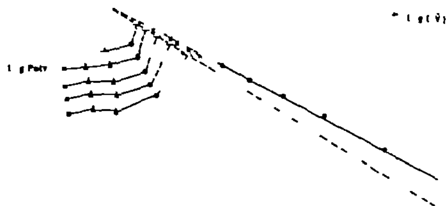


FIGURE 11 Same curves on logarithmi scales Note the straight lined appearance f the ascending curves The decrease in flow rates at the given volumes when Palv exceeds optimal values indicates negative ffort dependence

curves at the same elastic pressure rather than isovolume pressure-flow curves when evaluating the collapsibility of the airways Optimal conditions are defined as those corresponding to the flow maxima on the isovolume pressure-flow curves

Optimal pressure-volume curves, pressure-flow curves, and flow-volume curves of the different segments of the airway have been obtained by several methods The results of the different measurements agree except when obtained under conditions with great acceleration of flow and pressure where the findings indicate that the frequency response of the recording system is not adequate

At given values of the elastic pressure (Pel) of the lungs a decreased upstream resistance and a decreased collapsibility of the airway cause the optimal alveolar pressure to increase which is to be expected Similarly the optimal work done during the expiration of one vital capacity increases when the upstream resistance and the collapsibility of the airway decreases

The application of the function of normalization to the upstream segment of Mead et al (1967) during optimal conditions has shown that the upstream resistance is constant (s = 0) when the airway is flaccid but decreases during the expiration (s 0) when the airway is elastic and therefore able to resist collapse to a certain degree In this case the positive value of s is caused by an upstream motion of EPP as predicted in the previous chapter

The application of the function of normalization to the segment of Pride et al. (1967) during optimal conditions has shown that the resistance of this segment increases during the expiration (s 0) This is shown to be caused by compression of the collapsiblo segmont due to increasing negative critical transmural pressures The critical transmural pressure is defined as the pressure drop across the wall of the collapsible segment at the mo ment when the flow rate becoms maximal

The factors involved in the negative effort dependence have been evaluated The effect of compression of the air in the bellows is found to be negligible More important are flow dependent changes of the elastic properties of the airway due to compression of the downstream part of the collapsible egment followed by an increase in the upstream re i tance and in the collapsibility of the airway This has been demonstrated by means of a pitot static tube which allowed the relationship between the cross sectional area of the airway (A) and the transmural pressure (Ptm) to be determined This relationship defines the elastic properties of the airway at the tip of the pitot static tube It is straight lined but the position of the line changes when passing from optimal to supraoptimal conditions i e when the alveolar pres ure become greater than necessary for optimal flow Thi illustrates the change in the elastic properties of the airway when the negative effort dependence becomes apparent (Pedersen 1971)

The pitot tatic tube has also been used to determine the cros se tional area at EPP (Aepp) The ize of Aepp

thus determined agrees with that calculated on the assumption that the pressure drop from the inserted upstream stenosis down to EPP is entirely due to convective acceleration of the air

From the study of the upstream resistance of Mead _et al_ (1967) and Aepp it has been possible to describe _the motion of EPP in forced expirations with negative effort dependence_: during the initial part of the expiration EPP moves from the airway opening to the collapsible segment on its way upstream In the flaccid segment EPP does not proceed further but if the segment can resist a certain negative transmural pressure EPP moves further upstream until the upstream resistance is minimal at optimal alveolar pressures With increasing effort however EPP moves down to the collapsible segment again The degree of compression of this segment at EPP depends on its ability to resist collapse: _If the airway is flaccid_ the segment becomes *more and more compressed* at EPP; *the upstream resistance* of Mead _et al_ (1967) increases steadily during the expiration and may even be greater than that of the total airway before the compression starts The negative value of s in the function of normalization accordingly does not indicate a downstream motion of EPP but a decreased cross-sectional area at EPP _If the airway is elastic_ EPP moves upstream again during the last part of the expiration and ceases to exist when the elastic pressure of the bellows (Pel) becomes zero; the bellows

continue to empty only because the airway remains open as it can resist a negative transmural pressure

Based on the experience with the mechanical model a number of equations describing the flow conditions in the airway have been derived and solved by an analogue computer (table I and figure 12) It has been shown that it is possible to determine the critical transmural pressure from the flow rate and the constants describing the elastic properties of the airway (Pedersen 1972):

If we assume that the pressure drop in the airway of the mechanical model is mostly due to convective acceleration and orifice flow and if we further assume that the elastic properties of the airway can be described by the equation

4) $A = a\ (Ptm) + b$

the critical transmural pressure (Ptm_a) can be determined a

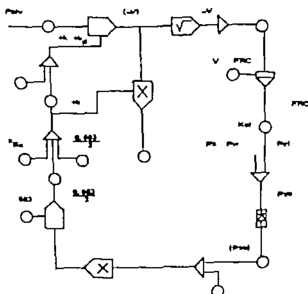
5) $Ptm_a = (1\ 206\ a)^{0\ 33} (\dot{V})^{0\ 67} - b$

A is the cross-sectional area of the collapsible airway when the transmural pressure is Ptm and $-\dot{V}$ is the maximal flow rate ($= -\dot{V}max$); a is the compliance of the airway and b is the cross sectional area when Ptm = 0

EQUATIONS DETERMINING THE AIRFLOW THROUGH THE AIRWAYS OF THE MODEL

1)	$(-\dot{V})^2$	$\frac{Palv}{k \quad k_w \quad k_d}$
2)	$-\dot{V}$	$\sqrt{(-\dot{V})^2}$
3)	V FRC	$(V_0 \quad FRC) \quad \int_0^t (-\dot{V})\, dt$
4)	Pel	Kel (V FRC)
5)	Ptm	Pel (Ps Pw)
6)	A	(Ptm) b
7)	A^2	A A
8)	$\frac{0.443}{A^2}$	$0.443 \quad \frac{1}{A^2}$
9)	k k_w	$\frac{0.443}{A^2} \quad \frac{0.443}{b^2} \quad k_{fix}$
[illegible]	k w	(w)
)	Ps Pw	(w)(-V)

TABLE I Equations determining the airflow through th airways f the model Explanation: $\dot{V}$ = flow rat Palv = pressure drop from the inside f the bellows to the airway opening k_s Ps ($\dot{V}$) where Ps is the pressure drop from the inside of th bellows to the point in the airway where the "waterfall begins (cf Prid et al 1967) k_w = Pw ($\dot{V}$)$^{-2}$ where Pw i the pressure drop across the "waterfall k_d = Pd ($\dot{V}$) ^{2} where Pd is the pressure drop from the "bottom of the "wate fall to the airway opening V is th volume f air in the bellows beyond FRC which is the volume when the elasti pressure Pel is zero K l is the elasticity of the bellows and springs Ptm is the transmural pressure f the collapsible airway (i the pressure inside minus the pressure outside) A is the cross-sectional area of the collapsibl airway a its complianc b its ross-sectional area when Ptm is z ro k_{fix} = Pfix ($\dot{V}$)$^{-2}$ where Pfix is the pressure drop acros the upstream stenosi (figure 7 no 6)



POTENTIOMETER

INTEGRATOR

ADDER OR INVERTER

MULTIPLIER OR DIVIDER
[illegible]

DIODE

FIGURE 12 Circuit diagram fo the analogue computer

These calculations show that flow limitation may occur even with dilated airways (Ptm > 0) when -V is sufficiently large (cf figure 13 unbroken curve -Vmax > 3 3 l/sec^{-1}) This indicates that the term dynamic compression of the airways may not be appropriate in the description of the mechanisms of flow limitation during forced expirations

The findings in the mechanical model and those calculated by the analogue computer agree fairly well (figure 13) We may therefore conclude that the events in the mechanical model for the major part are governed by the physical laws laid down in the equations solved by the computer

CHAPTER V

In this chapter the applicability of the new equations to normal and diseased lungs has been evaluated

From data in the litterature it has been possible to calculate the critical transmural pressures of different cross-sections of the tracheo-bronchial tree At large flow rates the critical transmural pressure is positive and first attained in the major bronchi which are then flow determining whereas the small bronchi are flow determining at low flow rates with negative critical transmural pressures

The equations have been evaluated in one normal subject and in a patient with asthmatic bronchitis From related values of optimal alveolar pressures (Palv) and maximal flow rates (Vmax) it has been

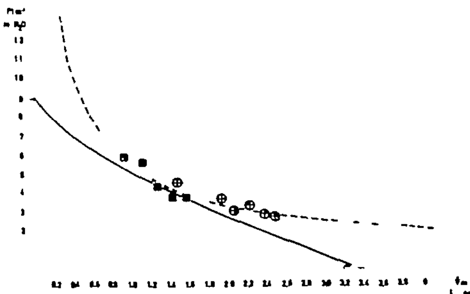


FIGURE 13 The relation hip between alculated and experimental values Unbroken curve alculated rel tionship from equation 5 in the text Cro ses in squares and circles point obtained from model experiments with large resp small re istance in the upstream stenosis (figure 7,no 6) Broken curve cal culated relationship from the experimental point using equation 1 in th text

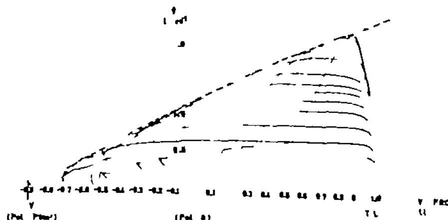


FIGURE 14 Flow-volume curves t varying degrees of ffort drawn by the analogue computer (cf tabl I and figure 12 k_{fix} 2 0 and k_d 0 cm H_2O 1 ^{2} sec^2 Kal = 10 cm H_2O 1 ^{1} = 0 134 cm^2(cm H_2O) ^{1} and b = 1 22 cm^2) As Pel = 10 (V FRC) the curves also how the relationship between Pel and V if the abscissa values are multiplicated by 10 The uppe borderline curve (broken) is the optimal flow-volume curve,or the MFSR curve f Mead et al (1967) if Pel is used as abs issa It is alculated from equation 7 in the text

demonstrated how the overall compliance of the airway (a) and the downstream resistance (k_d) can be determined This has been done on the assumption that the pressure drop from the alveoli to the flow determining segment is merely due to convective acceleration and that the optimal pressure drop from the flow determining segment to the airway opening can be considered analogous to the pressure drop across an orifice and hence expressed as $Pd = k_d\ (-V)^2$ The optimal pressure-flow equation will then be

$$6)\quad \frac{Palv}{(-Vmax)^{0\ 67}} = k_d\ (-Vmax)^{1\ 33} + \frac{0\ 603}{(1\ 206\ a)^{0\ 67}}$$

which is a straight line if we plot $Palv\ /(-Vmax)^{0\ 67}$ as a function of $(-Vmax)^{1\ 33}$ From this line k_d and a can be obtained

The relationship between the maximal flow rate (-Vmax) and the elastic pressure (Pel) describes the maximum flow static recoil (MFSR) curve defined by Mead _et al_ (1967) In terms of the new equations it has been pointed out that the sigmoidal shape of such curves obtained in humans can be explained by the effect of convective acceleration alone (_cf_ figure 14) The MFSR curve is defined by the following equation

$$7)\quad Pel = \frac{1\ 809}{(1\ 206\ a)^{0\ 67}}\ (-Vmax)^{0\ 67} - \frac{b}{a}$$

which is a straight line if we plot Pel as a function of $(-\dot{V}max)^{0\ 67}$ From this line a and b can be obtained

On the given assumptions it has thus been demonstrated that the flow resistive properties of the airway during a forced expiration can be described by the constants a b and k_d (cf Pedersen 1972)

The effect of bronchodilators on the shape of the isovolume pressure-flow curves has been evaluated in terms of changes of the constants a and b This evaluation is based on findings published in the litterature

The EPP concept has been found to agree with the concepts of the new equations The motion of EPP as described in the litterature can be explained by the location of the flow determining segments and their critical transmural pressures

The negative effort dependence has been discussed in terms of flow dependent changes in the constants a and b It is suggested that these changes may also cause transient increases of the maximal flow rate

Finally the influence of negative effort dependence on the estimation of the degree of bronchial compression from the flow curves has been discussed It is stated that an interpretation of initial overshoot of flow as representing th volume expelled due to collapse of the bronchi is dubious

References:

Briscoe W A & A B DuBois:
The relationship between airway resistance airway conductance and lung volume in subjects of different age and body size
J Clin Invest 1958 37: 1279-1285

Fry D L R V Ebert W W Stead & C C Brown:
The mechanics of pulmonary ventilation in normal subjects and in patients with emphysema
Amer J Med 1954 16 80-97

Mead J :
Mechanical properties of lungs
Physiol Rev 1961 41 281-330

Mead J J M Turner P T Macklem & J B Little
Significance of the relationship between lung recoil and maximum expiratory flow
J Appl Physiol 1967 22 95-108

Pedersen O F :
The cause of negative effort dependence in model experiments
Bull Physio-path resp 1971 7 327 331

Pedersen O F :
A new way to describe the mechanics of the expiration
Bull Physio-path resp 1972 8: 569-585

Pride N B S Permutt R L Riley & B Bromberger-Barnea
Determinants of maximal expiratory flow from lungs
J Appl Physiol 1967 23: 646-662

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 387

THE SMALL INTESTINE IN HYPOTENSION AND HEMORRHAGE

An experimental cardiovascular study in the cat

GÖTEBORG 1973

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 387

From the Department of Physiology and the Department of Surgery II
University of Göteborg Sweden

THE SMALL INTESTINE IN HYPOTENSION AND HEMORRHAGE

An experimental cardiovascular study in the cat

By

Ulf Haglund

Göteborg 1973

CONTENTS

This summary is based on studies reported in the following papers

I Reactions within consecutive vascular sections of the small intestine of the cat during prolonged hypotension
U Hoglund and O Lundgren Acta physiol scand 1972 84 151-163

II The effects of vasoconstrictor fibre stimulation on the consecutive vascular sections of the small intestine of the cat during prolonged regional hypotension
U Hoglund and O Lundgren Acta physiol scand 1972 85 547 558

III The effects of vasoconstrictor fibre stimulation on consecutive vascular sections of cat small intestine during hemorrhagic hypotension
U Hoglund and O Lundgren Acta physiol scand 1973 In press

IV Vascular reactions in the small intestine of the cat during hemorrhage
U Hoglund Acta physiol scand 1973 In press

V Mucosal lesions in the small intestine of the cat during low flow
C Åhrén and U Haglund Acta physiol scand 1973 In press

VI Cardiovascular effects of blood borne material released from the cat small intestine during simulated shock conditions
U Haglund and O Lundgren Acta physiol scand 1973 In press

The papers are referred to by their Roman numerals in the text

INTRODUCTION

Functional anatomy of the intestinal vascular supply

The intestinal vascular bed may be considered as a set of parallel-coupled circuits supplying the different layers of the intestinal wall Among them the mucosa has an unique position It follows from the specialized functions of this layer (i e secretion and absorption) that the metabolic demands are high as are the demands for blood as a transport vehicle According to Lundgren (1967) 60–80 per cent of the total intestinal blood flow is directed to the mucosa during resting conditions while 20 25 per cent of total intestinal blood flow is distributed to the villi (Svanvik 1973 a)

Every parallelcoupled vascular circuit consists of specialized sections coupled in series (Folkow 1967) Among them the exchange vessels the true capillaries constitute the key section The cardio vascular system is designed to provide the tissues with nutrients and remove products of cellular metabolism This all-important exchange between tissue and blood takes place across the thin walls of this vascular section The capillary itself exerts no active influence of either flow rate or on the exchange mechanisms of diffusion and filtration-absorption The vascular bed proximal to the capillaries can be divided functionally in two sections The precapillary resistance vessels mainly corresponding to arterioles are the main determinant of the amount of blood supply Changes in tone of this vascular section are reflected as changes in flow resistance The precapillary sphincters, considered to contribute relatively little to the total flow resistance influence the local flow distribution by determining the number of capillaries open for flow and hence the density of the perfused capillary network and the area available for transcapillary exchange Distal to the capillaries the vascular bed can also be divided in two sections The postcapillary resistance section contributes relatively little to the total resistance but is nevertheless very important since it affects the pre- to postcapillary resistance ratio Hence it greatly influences the mean capillary pressure and thus the important fluid movement between the intra and extravascular compartments The capacitance vessels mainly equivalent to the veins are capable to change greatly the regional blood volume with only small concomitant changes in flow resistance

The intestine in hypotension and hemorrhage

The vascular reactions of the small intestine during hypotension and shock have been described earlier by several authors using different

techniques and experimental models Concerning the resistance vessels both constriction and dilatation have been reported as earlier discussed at some length (III) For the capacitance vessels most authors agree that an intestinal pooling of blood occurs in shock (e g Wiggers 1950 Lillehei et al 1967) but also here exist contradictory reports (e g Swan and Nelson 1971) There exists however no previous study *where the reactions in all the intestinal consecutive vascular sections* particularly with respect to the neurogenic influence have been followed during hypotension or shock Such data are available from cat skeletal muscle (Lewis and Mellander 1962 Mellander and Lewis 1963) In this vascular bed the postcapillary (mainly capacitance) response to vasoconstrictor fibre stimulation is maintained much better during the course of hypotension than the precapillary (mainly resistance) one This causes a gradual fall in the pre- to postcapillary resistance ratio and hence an increase in mean hydrostatic capillary pressure from its initially reduced level which may ultimately even lead to an *outward filtration of fluid from the blood vessels late in hypotension* In the intestine with a surface area available for transcapillary filtration-absorption exchange some 10 times greater than that of the skeletal muscle vascular bed (Folkow et al 1963) such a chain of events might result in a disastrous loss of intravascular fluid On the other hand in case the neurogenic postcapillary (capacitance) response would rapidly decline during hypotension this might result in a considerable accumulation of blood within the intestinal vascular bed

Using a cross perfusion technique Lillehei (1957) showed that if the intestine was supplied with blood from a donor animal at normal perfusion pressure during a period of hemorrhagic hypotension the mortality rate was reduced from 90 to 10 per cent Moreover the hemorrhagic mucosal lesions regularly found in dogs dying in irreversible shock were prevented Lillehei suggested the existence of an intestinal factor in irreversible hemorrhagic shock and connected this factor to the hemorrhagic mucosal lesions Such lesions have since long time been known to occur in both man and experimental animals dying from various shock conditions (see e g Penner and Bernheim 1939 Wiggers 1950) The mucosal lesions were thought to be the result of an intense regional vasoconstriction (Klemperer et al 1940 Lillehei et al 1964 a b 1967) Subsequently the importance of the intraluminal content especially the enzyme trypsin for the development of these lesions has been stressed (Bounous et al 1964 a Bounous 1967)

Several authors have tried to identify the intestinal factor proposed by Lillehei (1957) Fine and coworkers (e g Schweinburg et al 1954 Fine et al 1959 Fine 1967) suggested that intestinal ischemia reflected in the mucosal lesions allows bacterial endotoxins from the intestinal lumen to enter the general circulation They proposed that

hemorrhagic shock becomes essentially a septic state which accounts for the irreversibility This concept as the sole explanation for irreversibility in shock has not been generally accepted (e g Carter and Einheber 1966 Kuida 1967) Moreover different vasoactive substances released from the intestine or the splanchnic area such as bradykinin histamine serotonin have been suggested to play an important role in the development of the irreversibility of hemorrhagic shock (e g Selkurt 1970) and also the myocardial depressant factor (Lefer 1970 Lefer and Glenn 1972)

The aim of the present study was to describe and analyze the reactions within the consecutive vascular sections of the intact small intestine during hemorrhage (IV) To elucidate the various factors involved it was considered necessary to approach this problem in a stepwise fashion by means of model experiments using regional (I II) or systemic hemorrhagic (III) hypotension in a denervated small intestinal segment (I) where however the vasoconstrictor nerve fibres could be stimulated intermittently (II III) With the knowledge thus achieved the more complex events taking place in the intact intestine in hemorrhagic hypovolemia could be analyzed allowing the defence mechanisms of the experimental animal to exert their full action (IV) In these series of investigations a technique closely related to that used in the abovementioned study on the skeletal muscle vascular bed (Lewis and Mellander 1962 Mellander and Lewis 1963) was utilized in order to facilitate a more close comparison between these two important vascular beds (II III IV)

Hemorrhagic mucosal lesions were regularly found in the small intestine of the experimental animals subjected to these periods of hypotension and hemorrhage A special study (V) was therefore devoted to these mucosal lesions where they were described microscopically and in addition their pathogenesis was investigated Furthermore in the abovementioned studies (I V) a rapid fall in arterial blood pressure was observed after restoration of the intestinal perfusion pressure in many cases leading to the animal s death within the first posthypotensive hour Such a chain of events was not reported in the studies in which the skeletal muscle vascular bed was subjected to hypotension indicating that the cat small intestine but not skeletal muscle releases material that causes a cardiovascular derangement This was examined further in a final study in which the importance of cardiac *versus* peripheral vasculo derangement as caused by the hypothetically released material was elucidated (VI)

METHODOLOGICAL CONSIDERATIONS

The methods used have been described fully in the different papers which should be consulted for details Below is only given a general survey

A Operative procedures The details of the operative technique were presented in paper I Briefly a segment of the small intestine weighing 30-70 g was isolated with intact vascular and nervous supply The greater omentum the spleen a major portion (70-80 per cent) of the pancreas the duodenum and the remainder of the small intestine were extirpated together with the colon The splanchnic nerves containing the vasoconstrictor nerve fibres to the small intestine were if desired cut and their distal ends placed on electrodes for graded electrical stimulation The adrenals could be denervated or entirely excluded from the circulation by ligatures For inducing local hypotension (papers I II V VI) an adjustable clamp was placed around the superior mesenteric artery (SMA) close to the aorta where the artery is not closely emeshed by nerves After heparinization one of the femoral arteries was cannulated to record systemic arterial blood pressure The other femoral artery was often cannulated for arterial blood sampling The intestinal arterial inflow pressure was recorded by means of a cannula in a major branch of the SMA distal to the adjustable clamp The superior mesenteric vein draining the intestinal segment and its lymph nodes was cannulated and after passing a drop recorder unit the blood was returned to the animal via a funnel connected to a cannula in the left external jugular vein inserted deep enough into the thorax to allow also an estimation of changes of the central venous pressure This arrangement allowed the venous outflow pressure from the intestine to be kept at any desired level and it was usually set at 10 mm Hg This pressure level is close to one present in the intact animal and furthermore it minimizes passive elastic recoil of the thinwalled veins (Öberg 1967)

In paper VI a left parasternal thoractomy was performed The cardiac sympathetic nerves (ansa subclavia) were divided close to the stellate ganglia and atropin (0 5-1 0 mg/kg b w) was given i v for a complete denervation of the heart A flow-probe for electromagnetic blood flow recordings was placed around the ascending aorta measuring cardiac output except the coronary flow Maximal dP/dt of the left ventricle (Linden 1968) was measured in some experiments via a cannula inserted through the left heart auricle

B Plethysmographic and radioactive isotope techniques The plethysmographic technique originally described by Mellander (1960) and

modified for the intestine by Folkow et al (1963) was used to follow changes in tissue volume and for determination of the capillary filtration coefficient (CFC) This technique was thoroughly described in paper I in which also the CFC determinations were discussed (see also Wallentin 1967) The jejunal segment and its lymph nodes were for this purpose enclosed in a triangular perspex plethysmograph filled with Tyrode s solution

In some experiments (I III IV) regional blood volume was followed concomitantly with tissue volume Red cells from the experimental animal were labelled with ^{51}Cr (AB Atomenergi Studsvik Sweden) according to the method described by Owen (1959) The γ-emission from the ^{51}Cr was recorded by a scintillation detector so collimated as to register radioactivity mainly from the tissues within the plethysmograph By using these two methods concomitantly it could be determined to what extent changes in tissue volume reflected a change in regional blood volume and/or in tissue fluid volume

C Acid-base balance of the experimental animal A 10 per cent glucose solution containing 10-20 mekv Na HCO_3 per 100 ml was slowly infused i v (0 10-0 15 ml/min) throughout the experiments in order to minimize the effects of the surgical trauma and anesthesia on acid base balance The amount of glucose infused did not induce any changes of the arterial plasma glucose level Hematocrit measured in some experiments did not change significantly

In papers I V in which all cats ventilated spontaneously arterial and intestinal venous blood pH was followed regularly throughout the experiments with an Astrup pH meter (pH-meter 27 with a micro electrode unit) and all prehypotensive control values were within the normal limits reported for the cat (Fink and Schoolman 1963) In most of these experiments arterial P_{CO_2} was followed and in some also arterial P_{O_2} Values suggesting a respiratory failure were never observed

The cardiovascular effects of the metabolic acidoses caused by surgery anesthesia and hemorrhage were studied in paper III In this paper a group of animals receiving the slow bicarbonate infusion was compared to another group with no such infusion The comparison showed that the metabolic acidosis was associated with a reduction in resistance vessel tone and a reduced vascular responsiveness to vasoconstrictor fibre influence These observations are probably explained by the well known vasodilatory effect of the hydrogen ion (Bygdeman 1963)

The cats used in paper VI were ventilated artificially To avoid lung atelectasis a slight hyperventilation was desired and tidal volume was set to 15 ml/kg b w and the frequency to 15/min A gas mixture containing 97 % O_2 and 3 % CO_2 was used Blood gas analyses were not performed in this series of experiments but the abovementioned

procedure has been shown to keep arterial P_{CO_2} within the normal values in other experiments of a similar nature in this laboratory (Thorén personal communication)

D Rheological considerations Changes in the rheological properties of blood known to occur during low flow states (e g Gelin 1956 Baeckström et al 1970 Appelgren 1972) must be minimized if the primary aim is to study vascular reactions per se For this reason 1-2 ml/kg b w of a dextran solution (1/3 Rheomacrodex R and 2/3 Macrodex R Pharmacia Sweden) was given per hour throughout the experiments Furthermore the tubings the funnel and the drop chamber were primed with the dextran solution before connecting them to the animals The total amount of dextran thus given was approximately equivalent to the doses (0 8 g/kg b w) given by Appelgren and Lewis (1972) to normalize the skeletal muscle microcirculation in dogs subjected to hemorrhage shock

E Histological technique Microscopic examination of the small intestinal mucosa was performed in some of the experiments included in papers II and IV and regularly in paper V At the end of the experiments tissue specimens were immediately cut out mounted on cork and fixed in 10% neutral formalin After preparation for routine embeddment the tissue was cut in sections about 4 nm thick mounted and stained according to v Gieson in hematoxyline-eosine and with McManus PAS stain In paper V the glasses were coded for unbiased histological examination

REACTIONS IN THE SERIES-COUPLED VASCULAR SECTIONS OF THE SMALL INTESTINE IN HYPOTENSION AND HEMORRHAGE

Activation of the sympathetic vasoconstrictor fibres to the small in testine by electrical stimulation of the isolated nerves or reflexly via e g the baroreceptors causes a characteristic response pattern of the series-coupled vascular sections (Folkow et al 1964 a b Öberg 1964) Upon stimulation of the constrictor fibres intestinal blood flow initi ally is reduced drastically (peak resistance response) and a marked constriction of the veins is also apparent However upon sustained stimulation intestinal blood flow again increases within 2-4 min reach ing a new steady state level that normally s only moderately re duced below prestimulatory control value while the neurogenic capa citance response remains essentially unaltered throughout the stimula tion period During the steady state phase of vasoconstriction the capillary filtration coefficient (CFC) reflecting the capillary surface area available for fluid exchange is considerably reduced sometimes to values below half of the control

A major aim of the present ser es of experiments was to investi gate the effect of hypotension on the above-mentioned neurogenic vascular adjustments in the small ntestine Such informat on was deemed necessary to be able to analy-e the much more complicated hemodynamic situation in the intact animal during hemorrhage To approach this question four different types of experiments were per- formed in which the react ons of the series coupled vascular sections were followed In paper I perfusion pressure to a denervated segment of the small intestine was lowered by partial occlusion of the superior mesenteric artery without any activation of the reg onal vasoconstric tor fibres In papers II and III the same preparation was used but now the reg onal vasoconstrictor fibres were ntermittently st mulated either during regional hypotension (II) or during generalized hypotension caused by hemorrhage (III) Finally in paper IV the hemodynamical adjustments occurring in the innervated small intestine were followed after hemorrhage marked enough to induce hypotension The analyses of the latter more complicated situation was then facilitated by the information gained from the previous stud es

Experiments were performed at different levels of blood pressure (I II III) or w th the an mals exposed to d fferent degrees of hemorr hage (IV) However the s bsequent description and discussion of the results w ll deal mainly with cats subjected to the most marked degree of regional hypotension o to the most severe hemorrhage since the vascular reactions seen during such circumstances were more pro nounced and hence more easily analyzed

ditions and was not normalized after restoration of the perfusion pressure (II III)

In the innervated intestine a rapid reduction of the intestinal blood content (3-4 ml/100 g) was recorded upon bleeding (IV) compared to a reduction of 0 5 1 ml/100 g in the denervated intestine when bled to the same arterial pressure level (60 mm Hg, Haglund and Lundgren unpublished data) *During the final hour of hemorrhage the regional* blood content again increased 1-1 5 ml/100 g reflecting a moderate pooling of blood After retransfusion intestinal blood volume returned rapidly to the prehypovolemic control level A further augmentation of blood content beyond control was recorded during the first 15-30 min after retransfusion amounting to 0 5 1 ml/100 g

Discussion

It is generally agreed that the peripheral vasoconstriction following hemorrhage is predominantly caused by a reflex activation of the sym *pathico-adrenal system* (IV *for review see* Chien 1967) *The enhanced* sympathetic rate of discharge seems to be largely unchanged throughout a hypotensive period (Lundgren et al 1964) although a final decrease in the firing rate might occur just prior to death (Lundgren et al 1964 Chien 1967) Recently however McNeil et al (1970) suggested that *the sympathico-adrenal system plays only a minor role in explaining* the resistance reactions of the intestinal vascular bed upon hemorrhage and proposed that vasopressin and angiotensin were instead of great importance As discussed at some length in paper IV the experimental basis for their suggestion can be questioned on several points Further*more experimental observations made in the present series of experi*ments strongly argue against the views of McNeil et al Although one cannot entirely exclude that the mentioned humoral factors might contribute to the vascular changes occurring during hemorrhage the present results strongly suggest that their contribution is minor (compare e g *the results in papers III and IV*)

Thus the vascular reactions observed in the present series of ex periments will be discussed with regard to the competition between the neurogenic constrictor fibre influence and the local control mechanisms The remote neurogenic control of the small intestinal vascular *bed of the cat is only constrictory* (cf Kewenter 1965) *and the con* striction added by catecholamines released from the adrenals is normally of relatively minor importance (Celander 1954) Nervous vasoconstric tion may be counteracted by a local accumulation of dilating metabolites and low oxygen tension as induced e g by ischemia (Cobbold et al 1963 *Lewis and Mellander 1962 II see below*) *Furthermore* the inherent properties of the myogenically active vascular smooth

muscles to modulate their activity and consequently average length to stretch represents another local mechanism for vascular control (e g Folkow 1964 Johnson 1964 for further references see I)

The term vasodilator metabolites deserves some further comments It is commonly used to cover all those changes thus also decrease of oxygen tension - in local chemical environment of the vascular smooth muscle cells that tend to cause their relaxations (Lewis and Mellander 1962 Cobbold et al 1963 I II III) It seems likely that vasodilator metabolites represent a whole series of substances and local chemical changes some of which probably are still unknown and which may vary inbetween different tissues It is however well estab lished that tissue acidosis caused by an increased tissue P_{CO_2} and or lactic acid production causes a vasodilatation This was illustrated also in the present series of experiments (III; Fig 5) However a marked intestinal vasodilatation was seen during and after prolonged hypotension even when arterial plasma pH was kept within normal values by infusions of bicarbonate (I II III) Hence other contribut ng factors must exist such as low tissue P_{O_2} Increases of tissue potassium concentration and tissue osmolarity The two latter factors were how-ever not found to be significantly increased in the intestinal venous plasma of the present experiments (Fara and Haglund 1973) Further a release of vasoactive local hormones may contribute and it has been shown that the intestine after periods of local or he iorrhagic hypo-tension releases considerable amounts of serotonin (Fara and Haglund 1973) which has been shown to be a potent intestinal vasodilator (Biber et al 1973) The reason for the serotonin release in hypotension is not clear but it may be a consequence of the derangement of the mu cosal tissue (V see also below) The tissue derangement ultimately resulting in gross destruction as studied in the experiments reported in paper V probably implies that a number of substances are released that are not normally involved in the physiological local chemical control of the vascular smooth muscles

Upon bleeding an animal with intact innervation of the intestine the intestinal resistance vessels were constricted as arterial pressure fell to 70 mm Hg In the denervated intestine (I II III) such a re duction of arterial pressure caused a rapid reduction in flow resistance probably mainly reflecting the myogenic autoregulatory properties of the intestinal vascular smooth muscles In the innervated intestine on the other hand the sympathetic vasoconstrictor activity overruled this local dilator mechanism and was probably facilitated in doing this by the reflex respiratory alkalosis induced by bleeding (IV) However after 60-90 min of more severe blood loss arterial blood pressure and intestinal flow resistance both started to fall This gradual d crease of intestinal flow resistance was probably secondary to an accumulation of local vasod lator factors a d in the cats considered to be in shock

resistance never normalized even after retransfusion (IV)

As mentioned in the introduction and discussed at some length in paper III there are conflicting reports in the literature concerning the reactions of the intestinal resistance vessels after hemorrhage Below only such studies in which the intestinal blood flow and flow resistance have been followed for at least one hour of controlled hypotension will be discussed Using the original Wiggers method of standardized experimental shock (Werle Cosby and Wiggers 1942 Wigger 19,0) on dogs with intact intestinal innervation Selkurt *et al* (1947) Cull *et al* (1956) and Selkurt and Brecher (1956) recorded an elevation of intestinal flow resistance early after hemorrhage but during the course of the hypotensive period resistance fell to or below control Upon retransfusion flow resistance was initially below control but rose then to or above control *i e* a response pattern similar to that found in the present study (IV) A similar hemodynamic readjust ment was also reported by Chalmers *et al* (1967) bleeding rabbits 26 per cent of the total blood volume Furthermore if the impact of the reflex sympathetic vasoconstriction on the intestine was eliminated by cutting the nerves no initial resistance elevation took place and the subsequent fall in flow resistance was accentuated (*cf* III)

There are however reports that are contradictory to the findings of the present study For example Selkurt (1958) and Friedman (1961) using modified Wiggers techniques in the dog registered no or only slight changes in flow resistance during and after hemorrhagic hypo tension Furthermore Lillehei and coworkers (*e g* Longerbeam *et al* 1962 Lillehei *et al* 1964 a b) bleeding dogs to 35-40 mm Hg and keeping this pressure level for 4 hours found an increased intestinal flow resistance and blood flow amounted to only 10-20 per cent of control Increased flow resistance was reported also by Dedichen (1972) on dogs and by Barton *et al* (1972) in baboons in investigations with the Wiggers technique

The above mentioned reports on the reactions of the resistance vessels during and after hemorrhage are contradictory and conflict in part with the results of the present study The great variation in ex perimental models (*e g* in pressure level and length of hypotensive period chosen) the varying tolerance to blood loss between different experimental animals and species of animals make strict comparisons very difficult if not impossible Furthermore the resistance responses are not merely the result of luminal changes f the resistance vessels since changes in the rheological properties of the blood also contribute and are likely to vary in extent depending on species extent of trauma, experimental procedures etc The difficulties are also illustrated by the findings reported in paper IV in which one and the same standardized bleeding procedure induced rather different effects on *e g* arterial blood pressure which in that study suggested the division of the

material into a shock and a non-shock group The observed difference was ascribed tentatively to a disturbance of the acid base balance (IV) but other factors were probably also involved

The magnitude of the capillary filtration coefficient, CFC is related to the size of the capillary surface area perfused and to the number of pores per unit capillary surface area (Pappenheimer 1953 Mellander 1960 Cobbold et al 1963 Wallentin 1967) As capillary permeability is reported to be unaffected by periods of low blood flow and hypoxia (Korner 1959 Arturson and Thorén 1965 Scott et al 1967) changes in CFC reported in the present series of experiments in all likelihood reflected alterations in the capillary surface area available for fluid exchange The size of this area in turn is considered to be governed by the average activity of the sphincter section of the precapillary vessels (Cobbold et al 1963) Thus the CFC changes reported in the present study are attributed to reactions in the precapillary sphincters CFC remained unchanged or was elevated slightly (shock group) upon bleeding intact cats (IV) It is well established that the intestinal CFC decreases upon sympathetic activation during control conditions (Folkow et al 1964 b, see also II III) The rapid CFC increase seen in the face of a neurogenic constriction of the resistance vessels upon severe blood loss and hypotension indicates that local dilator influences on the precapillary sphincters (I) can then overwhelm the influence of the sympathetic vasoconstrictor fibres This response probably reflected the accumulation of tissue metabolites and local hypoxia since the precapillary sphincters seemed in this situation to be more dominated by the local chemical environment than to transmural pressure variations (I)

The capacitance vessels constricted rapidly upon bleeding This reduction of intestinal blood volume probably reflected an active neurogenic constriction as well as a passive constriction caused by elastic recoil secondary to a decrease of transmural pressure as discussed in paper IV (see also Öberg 1967) In the late phase of severe hypovolemia and hypotension and during the early phase of the post hypovolemic period a regain of tissue volume occurred Part of this tissue volume increase was due to some accumulation of blood in the intestinal capacitance vessels due to a failing constrictor fibre influence but the major part of the volume increase reflected an increase of tissue fluid (see below) The moderate extent of blood pooling was probably also due to the accumulation of local vasodilator substances counteracting the neurogenic constriction of the capacitance vessels as was also suggested by the results obtained in the model experiments (II III)

A marked intestinal congestion i e a net increase of regional blood volume after periods of hemorrhagic hypotension has been reported by several authors using the dog as the experimental animal

(e g Selkurt et al 1947 Wiggers 1950 Reynell et al 1955 Lillehei et al 1964 a b Robinson and Mirkovitch 1972) This has also been reported from the rat (Robinson et al 1966 Bacalzo et al 1971) and in addition in some experiments performed by Abel et al (1965) in the monkey Several authors have monitored portal venous pressure during and after hemorrhagic hypotension to obtain indirect evidence for an accumulation of blood in the intestinal capacitance vessels Among them Cull et al (1956) and Friedman (1961) reported an increase of portal venous pressure during hypotension as well as after retransfusion In the latter phase of shock also Wiggers et al (1946) Selkurt et al (1947) Selkurt and Brecher (1956) Johnson and Selkurt (1958) and Selkurt (1958) found an elevated portal pressure in the dog and the same finding was reported in the monkey by Barton et al (1972) Changes in portal venous pressure are however not invariably related to changes in the intestinal vascular bed but are also greatly dependant upon the intrahepatic flow resistance and/or upon a falling heart On the other hand a more direct approach to study venous tone was used by Alexander (1955) whose results suggested that a relaxation of the capacitance vessels can ensue in severe hemorrhagic hypotension

From this survey of the literature it is evident that most studies including the present one indicate that a certain pooling of blood can occur in the intestinal capacitance vessels However the volume pooled in the intestinal vessels was comparatively small i e not more than 1-2 ml in a 3 kg cat (approximately 100 g intestinal tissue) Such a small reduction of the circulating blood volume approximately 1 5 per cent of the total volume can by no means be crucial for the development of irreversible shock as has been suggested by Lillehei et al (1964 a b 1967)

In the late phase of hypotension and in the posthypotensive period an increase of tissue volume was observed which could not be ascribed to an augmentation of the regional blood content (I-IV) Secretion and/or transcapillary fluid filtration (oedema) may then be involved Although no experiments have been performed to eliminate active secretion as contributing to the tissue volume augmentation it seems less plausible that such an energy consuming process like secretory work would be induced when intestinal blood flow was lowered in some experiments below half the control Moreover this increase of tissue volume was not affected by giving the animals atropin which would block such secretion that is cholinergically controlled It seems more likely that the observed tissue volume augmentation was caused by a deranged Starling equilibrium across the capillary wall Such a disturbance may be induced by a deterioration of the autoregulatory properties of the intestinal vascular smooth muscles which seem to be designed mainly to keep mean capillary pressure constant (Johnson 1964)

and the finding was first interpreted solely in terms of an elevated mean capillary hydrostatic pressure (I II) However when the severe destruction of the villi in these experimental situations was revealed (V) a disturbance of the Starling equilibrium caused by an increase in the extravascular colloid osmotic pressure was proposed at least as a contributing factor This would also explain why fluid accumulated in the lumen It is however not possible to distinguish between these two mechanisms with the present techniques

Similar findings of an increased tissue volume during hemorrhagic hypotension was reported by e g Johnson and Selkurt (1958) who measured intestinal weight These authors interpret their data as mainly caused by the accumulation of fluid in the gut lumen In agreement with the present results (III IV) An accumulation of fluid in the lumen was also reported by Cook et al (1971) and it is possible that such an accumulation of luminal fluid is reflected clinically by the bloody diarrhea during shock reported by many authors (see e g Penner and Bernheim 1939 Wigger 1950 Lillehei et al 1964 a b Ming 1965; see also next chapter) However the maximal volume lost into the small intestine in the present experiments amounted at most to 2 3 per cent of the intravascular volume and cannot possibly be of any great significance in explaining the irreversibility" of the observed shock situation (see above)

THE INTESTINAL MUCOSAL LESIONS

In the present experimental series macroscopic hemorrhagic mucosal lesions were found regularly in the small intestine except in the experiments of paper I in which local hypotension was induced in a sympathetically denervated intestinal segment As mentioned in Introduction similar lesions have been reported earlier by many authors in connection with autopsies after various forms of shock (see e g Dupuytren 1835 Billroth 1867 Penner and Bernheim 1939 Ming 1965 Sörensen and Vetner 1969 Hugon and Bounous 1971 for further ref see Marston 1962 a Bounous 1969) Such lesions are also a characteristic finding in dogs dying in shock (Wiggers 1950 Lillehei 1957 Bounous 1967) and they have been reported too in studies on rats (Robinson et al 1966 Ochsenfahrt and Winne 1969 Bacalzo et al 1971) but have not been described previously in the cat

As regards the pathogenesis of these lesions Penner and Bernheim (Penner and Bernheim 1939 Klemperer et al 1940) suggested that they were of ischemic origin These authors proposed that the severe reduction of mucosal-villous blood flow was secondary to an intense vasoconstriction of the mucosal vessels caused by overactivity of the sympathico-adrenal system This view has been supported by among others Lillehei and coworkers (see e g Lillehei et al 1964 a b 1967) In the last decade it has also been suggested that the intestinal contents especially the presence of pancreatic trypsin should be of prime importance for the mucosal lesions (Bounous et al 1964 a 1967 Bounous 1967) According to this view hypoxia makes the epithelium vulnerable to tryptic digestion

The hemorrhagic mucosal lesions of the cat small intestine observed in the present series of experiments were described microscopically in paper V In that study segments of the small intestine were all subjected to a 2 h period of hypotension at 30 mm Hg with a concomitant electrical stimulation of the regional vasoconstrictor fibres at 6 Hz Experimental procedures were also undertaken aimed at preventing the development of the lesions such as perfusing the lumen of the different intestinal segments with either oxygenated or nitrogenated saline or by installing glucose or saline into the gut lumen (V) A new hypothesis concerning the debated pathogenesis of the mucosal lesions was also presented (III V)

Results

The cardiovascular events recorded in paper V were largely similar to those reported previously (I-IV) Briefly after a short lasting constriction of the intestinal resistance vessels upon reduction of the perfusion

pressure with concomitant activation of the vasoconstrictor fibres Intestinal blood flow and flow resistance levelled off at largely the same low levels as described in the previous experiments (I IV) Upon restoration of perfusion pressure and elimination of the vasoconstrictor fibre influence intestinal flow resistance was normalized while regional blood flow was below control due to a falling arterial blood pressure In the posthypotensive period arterial blood pressure fell rapidly leading to the death of many of the animals Despite the reduction in perfusion pressure intestinal blood flow increased reflecting a decreased regional resistance to blood flow

The macroscopic appearance of control segments showed hemorrhagic lesions in the mucosa in 8 of 9 cats The lesions varied from petechial bleedings to mucosal destruction of the entire segment The segments that had their lumen continuously perfused with nitrogenated saline or filled with saline/glucose or flushed with saline showed the same type and extent of mucosal lesions while the macroscopic appearance of the segments slowly perfused intraluminally with oxygenated saline were normal in 6 out of 7 segments only one showing petechial bleedings

All the segments were also coded for microscopic examination A grading system for defining the extent of the lesion proposed by Chiu *et al* (1970) in studies on dogs was applied (V Fig 1) It was clear from this examination that the segments slowly perfused intraluminally with oxygenated saline were normal or only slightly damaged (grade 0-II) while the others exhibited similar kind and extent of lesions as those used as hypotensive controls These segments showed profound mucosal lesions often with denuded villi and total disintergration of the villous lamina propr a (grade III V) There were no microscopic lesions or even perivascular inflammatory cell infiltrations in any other layer of the intestine except the mucosa

Discussion

As mentioned in Introduction, mucosal lesions have been reported in both man and experimental animals dying in various types of shock including hemorrhag c shock The pathogenesis of these lesions is how ever debated and most investigators have been inclined to ascribe them to a severe reduction in blood supply as a result of reflex vasoconstriction Whichever the case indirect evidence points to a hypoxic origin (Bounous et al 1963 1964 a b Ahonen et al 1970 Brown et al 1970) a view that is further substa tiated by the present finding since the addition of only a limited supply of oxygen via the intestinal lumen can prevent the villous lesions A similar finding was reported by Robinson et al (1966) who were able partly to prevent the mucosal

lesions seen after half an hour of complete intestinal ischemia by intraluminal oxygen perfusion Further the hypothesis that the intestinal contents especially the pancreatic enzyme trypsin should be of great importance for the villous lesions (Bounous et al 1964 a Bounous 1967) is not supported by the present findings in which the studied intestinal segments were disconnected from the pancreas and where the development of the mucosal lesions was unaffected by luminal flushing with saline or perfusion with saline during the hypotension as long as the luminal perfusate did not contain any oxygen These observations are in agreement with those of Cook et al 1971 It can be concluded therefore that hypoxia per se is the key factor in the pathogenesis of the mucosal lesions and that they easily develop even in the absence of trypsin

As discussed in paper V the villous mucosal lesions have the same microscopical appearance in man dog cat and rat They are noticed first at the tips of the villi (Robinson et al 1966 Bounous 1967 1969 Chiu et al 1970 Cook et al 1971 V) which indicates that this region suffers most severely from hypoxia It is however not clear why the villi should be so hypoxic as to cause tissue destruction since total intestinal blood flow was in the present experiments not reduced more than about 40 per cent of control and often not that much in the experimental situations where mucosal lesions regularly ensued (II-V) Further "villous blood flow is unchanged or only slightly reduced during periods of local hypotension (Lundgren and Svanvik 1973) at pressure levels similar to those maintained in paper V Furthermore villous blood flow is even somewhat increased upon stimulation of the vasoconstrictor nerves at normal pressure levels (Svanvik 1973 b) Moreover in some experiments it has been demonstrated that the same degree of hemorrhage as used in IV did not induce any significant reduction in "villous plasma flow (Haglund Lundgren and Svanvik to be published) Thus neither regional hypotension nor hemorrhage seemed to induce any significant reduction of villous blood supply and yet hypoxia and tissue lesions developed only in this part of the intestine

It is of course not surprising that the villi become hypoxic in situations of a total obstruction of intestinal blood flow However hypoxia in the face of an almost unchanged villous blood flow is more difficult to understand In papers III and V a hypothesis explaining the apparent paradox was presented (see also Haglund et al 1973) This theory is based on the existence of a countercurrent exchange mechanism between the central arterial vessel and the subepithelial network of capillaries of the villi (Lundgren 1967) This mechanism implies that substances reaching the villi via the arterial blood stream tend to diffuse from the central arterial vessel to the nearby subepithelial capillary network whenever an arterio-venous concentration

difference is at hand It was shown by Kampp et al (1968) that oxygen which easily passes the vascular endothelium to some extent was short circuited in such a way in the villi even at normal blood flow In severe regional hypotension mean transit time in the villi has been shown to be increased up to 5 times that in the control situation (Lundgren and Svanvik 1973) In this situation the chances for extravascular shortcircuiting of oxygen in the villi is much increased and particularly the villous tips may then become virtually anoxic despite an almost unchanged volume flow of blood It is therefore proposed that the villous lesions observed in paper II III V and VI were caused by a strictly local tissue hypoxia secondary to a greatly enhanced countercurrent exchange diffusion of oxygen as a result of a hemodynamic situation characterized by a low pressure head wideopen villous blood vessels and a, consequently greatly reduced linear flow rate Such a retardation of linear flow rate of blood was also observed in the villous vascular loops during hemorrhage (Haglund Lundgren and Svanvik to be published) explaining the mucosal lesions seen in the experiments of paper IV

The currently most accepted hypothesis for explaining the intestinal mucosal lesions in shock implies that a pronounced vasoconstriction of the mucosal vessels occurs during e g hemorrhagic hypotension (Lillihei et al 1967) Recently this adrenergic theory of shock has been questioned (Jacobson 1972 Swan et al 1972) and the countercurrent hypothesis outlined above does not necessarily imply any mucosal vasoconstriction However it has been claimed that only experimental findings obtained in shock experiments on subhuman primates can be extrapolated to human (Swan et al 1972) This may hold true for the endotoxin shock in which the hemodynamic readjustments differ markedly e g between dog and baboon However as regards hemorrhagic hypotension the mesenteric vascular reactions are largely similar in cat dog and baboon Furthermore intestinal mucosal lesions have been demonstrated in man after hemorrhagic hypotension (Marston 1962 a b Ming 1965 Hugon and Bounous 1971) and some observations seem to suggest that an intestinal countercurrent exchanger exists in the human small intestine (Hultén et al 1972)

As discussed in the previous chapter intestinal tissue volume increased in the latter half of the period of hypotension and more often so in the post-hypotensive period (I–IV) In the experiments described in III and V the increased tissue fluid volume was to a great extent found in the intestinal lumen Such an accumulation of intraluminal fluid was evident only after local hypotension (I II VI) although the amount was not estimated in these experiments The mechanism inducing the augmented tissue fluid volume has already been discussed to some extent above (see also I–V) and it was suggested that the Starling equilibrium across the [illegible] had become deranged The deterioration at the capillary level is [illegible] be caused at least partly by an increased tissue colloid [illegible] pressure secondary to protein leakage from destroyed villous cells The [illegible]

causal relationship between the mucosal lesions and the development of irreversibility in shock as first suggested by Lillihei (1957) will be discussed in the next chapter

The macroscopic appearance of the intraluminal fluid was that of tissue fragments mixed in partly bloody fluid a picture easy to correlate to the microscopic appearance of the mucosal lesions (V Fig 1) Bloody diarrhea has often been reported in experimental shock studies on dogs in which the survival time of the animals was sufficient for the diarrhea to develop (see e g Wiggers 1950 Lillihei 1957 Lillihei et al 1964 a b) and it is not uncommon in clinical conditions in which mucosal lesions have been described (e g Penner and Bernheim 1939 Ming 1965 Sörensen and Vetner 1969) The fluid accumulating in the intestinal lumen of the present experiments may therefore correspond to the fluid of the bloody diarrhea observed in patients in shock

GENERAL CARDIOVASCULAR EFFECTS INDUCED BY SIMULATED SHOCK CONDITIONS IN THE SMALL INTESTINE

The existence of an intestinal factor in irreversible hemorrhagic shock was proposed by Lillehei (1957) and since then various substances have been suggested to be released from the splanchnic area in the shocked animal (see e g Fine 1967 Bounous 1969 Selkurt 1970 Lefer 1970 Lefer and Glenn 1972) In the present series of experiments it was observed that the experimental animals subjected to local hypotension at 30-35 mm Hg for 2 hours seemed to be in a good general condition during the hypotensive period However after releasing the partial occlusion of the superior mesenteric artery a rapid fall in arterial blood pressure started leading to the death of many animals within one hour (I II V) A similar chain of events was also observed after periods of general hemorrhagic hypotension (III IV) As local changes i e venous pooling of blood and/or accumulation of fluid in the intestinal lumen never were of such an order of magnitude as to cause a significant reduction of the total blood volume (see above) a release of material from the gut causing a cardiovascular derangement seemed likely (I V) Experiments aimed at obtaining further evidence for the release of toxic substances from the intestine as well as analysing their mode of action, were presented in paper VI

Results

The intestinal vascular reactions during and after local hypotension with concomitant stimulation of the vasoconstrictor nerves were similar to those described earlier (I-IV) and will not be further commented on here Mucosal lesions and an intraluminal accumulation of fluid were evident in all these experiments The heart of the animal was completely denervated to be used as an indicator of possible substances in the blood stream exerting any direct influence on myocardial contractility

Upon releasing the partial occlusion of the superior mesenteric artery and ending the electrical stimulation of the vasoconstrictor fibres arterial blood pressure declined rapidly and th s reduction was paralleled by a fall in stroke volume in external cardiac work and in maximal dP/dt under circumstances where central venous pressure tended to rise Heart rate remained constant because of the cardiac denervation while total peripheral resistance was elevated above control After a similar flow reduction of the skeletal muscular vascular bed no such signs of depressed cardiac function were noticed In another series of experiments the intestinal venous outflow was collected in a plastic beaker during the first 4-5 posthypotensive min and simultaneous-

ly substituted with an equivalent blood amount from a healthy donor animal In such experiments no significant change in arterial blood pressure in stroke volume or in external cardiac work were detected However when the shed intestinal venous blood was returned to the animal in a second exchange transfusion cardiac performance and arterial blood pressure fell rapidly Further administration of venous *plasma from a shocked intestine to a healthy animal led to a de* crease in maximal dP/dt and in blood pressure while left ventricular end diastolic pressure was unchanged or slightly elevated

Discussion

The results obtained in the present series of experiments (I–VI) strongly favour the opinion that the cat small intestine releases material into the blood during and particularly after a prolonged period of severe *regional or hemorrhagic hypotension, and that this material* has a depressant effect on cardiac function by reducing cardiac contractility (VI) The latter effect has been demonstrated convincingly also on an isolated working rat heart preparation (for technical details see Isaksson 1972) To the recirculating system of such a preparation contain ing 35–40 ml perfusate an addition of 2 ml of intestinal shock plasma induced a deterioration of heart performance within 2 3 min as reflected in a falling cardiac output and left intraventricular pressure when the left end diastolic pressure was kept constant Intestinal control plasma had not such effects (Haglund Isaksson and Lundgren to be published)

A great number of investigators have searched for the intestinal factor in hemorrhagic shock proposed by Lillehei (1957) Fine and coworkers (see e g Schweinburg et al 1954 Fine et al 1959 Fine 1967) have suggested that all forms of shock are complicated by the absorption of endotoxins from an ischemic intestine and that the follow ing endotoxemia is the important factor However this view is far from generally accepted (cf Lillehei et al 1964 a b Carter and Einheber 1966 Kulda 1967) The acute effect of endotoxemia on the cat is a rapid and very pronounced pulmonary vasoconstriction leading to a large rise of right atrial pressure (Kulda et al 1961 Greenway and Murthy 1971) Central venous pressure was never elevated to that extent in the present series of experiments Further according to Hinshaw et al (1972) blood from endotoxin treated dogs does not depress cardiac work performance per se

Several vasoactive substances have been proposed to be released from the intestine after periods of total intestinal occlusion or hemorr *hagic hypotension (see e g Selkurt 1970 and also paper VI) In a* recent study from this laboratory a release of vasoactive material from the small intestine into the venous plasma was observed after periods

of local and hemorrhagic hypotension (Fara and Haglund 1973) At least the main part of this vasoactivity was shown to be caused by serotonin released in great amounts from the intestine during prolonged severe hypotension It is however not likely that serotonin contributes to the depressed cardiac function in the present experiments as discussed in paper VI In the study of Fara and Haglund (1973) it was also demonstrated that osmolarity and potassium concentration in intestinal shock plasma was normal

Lefer and coworkers have proposed a release of a myocardial depressant factor (MDF) during various shock conditions (see e g Lefer 1970 Lefer and Glenn 1972) thought to be a polypeptide of a molecular weight around 800–1000 and released from the pancreas during ischemia and hypoxia According to this hypothesis pancreatic lysosomal enzymes should induce the formation of MDF which should reach to blood via the thoracic lymph duct (Lefer 1970 Lefer and Glenn 1972) For several reasons it is unlikely that MDF contributed to the cardiac depression seen in the present series of experiments First the major part of the pancreas was extirpated and the [illegible] small part was in the experiments reported in papers I II [illegible] perfused at normal arterial blood pressure Second the cardiac depression in the present experiments started within 1 min after the [illegible] of intestinal perfusion pressure (VI) while the effects of MDF seem to appear first after some 60 min upon injection Third the present myocardial depressant effect was obviously caused by material in the intestinal venous blood (VI) while MDF is transported from the pancreas via the lymphatics

A factor depressing cardiac performance was also reported by Williams et al (1969 a b) They found a rapid [illegible] output during as well as after a 2 hour period of [illegible] mesenteric artery occlusion This cardiac effect could be [illegible] by intestinal extirpation Williams and coworkers also [illegible] causing depression of cardiac performance in portal venous [illegible] taken during and after the intestinal ischemia [illegible] heart papillary muscle from cats previously subjected [illegible] these authors record the greatest decrease in [illegible] However output) during the 2 hour period of complete [illegible] struction while other authors usually have reported [illegible] are in a good general condition during this phase [illegible] (see e g Milliken et al 1965) and that hypo[illegible] delayed if the obstruction is not released (Sh[illegible] 1959) [illegible] furthermore difficult to understand how a depressant factor reaches the heart in a situation in which the blood circ[illegible] organ is entirely blocked It seems difficult [illegible] the release [illegible] et al really have provided evidence for the [illegible] of intestinal material causing myocardial depression since the [illegible] of [illegible]

shocked cat papillary muscle assay method used can be questioned

The release of lysosomal enzymes (acid phosphatase ribonuclease cathepsins) from an ischemic gut has been reported by several authors (Sutherland et al 1968 Bounous and McArdle 1969 Röttger and Oran 1969 Glenn and Lefer 1971 and Robinson and Mirkovitch 1972) In some of these experiments complete intestinal ischemia has been induced by occluding the supplying artery It is believed that the release of lysosomal enzymes is a consequence of ischemic tissue injury (Bounous and McArdle 1969) Moreover infusion of lysosomal enzymes to healthy dogs depresses cardiac function promptly (Glenn et al 1972)

From the discussion above it is evident that several substances have been proposed to be released from the intestine during and after shock As discussed in paper VI it seems also unlikely that there should exist only one key substance or mechanism causing irreversibility in shock It seems reasonable however to assume that the severely injured intestinal mucosal tissue releases products of tissue destruction such as local hormones polypeptide-like substances and various enzymes among them lysosomal ones capable of impairing cardiac function In the model experiments presented in paper VI this material was injected more or less directly into the right atrium by-passing the liver which probably facilitated the rapid and often fatal effect of the intestinal venous blood The liver may be able to protect the animal from this toxic material at least to a certain extent (cf Glenn et al 1972) On the other hand the liver also suffers from ischemia during hemorrhage and the protective capacity in such a situation may be much reduced (Selkurt 1959)

In hemorrhagic shock as it is encountered in clinical medicine cardiac depression caused by material released from the small intestine is probably only one of several contributing factors such as pooling and stagnation of blood shifts of interstitial fluid and biochemical deterioration in the cells causing irreversibility (Rush 1972) The quantitative importance of these different factors depends probably on the circumstances In certain clinical situations as after embolectomy in the superior mesenteric artery and after restoring blood flow to a strangulated small intestinal segment cardiac depressing material from the intestine may be of great importance in explaining the shock situation which often ensues after these procedures (Marston 1971)

SUMMARY AND CONCLUSIONS

1 The series-coupled vascular sections of the cat small intestine were studied using a plethysmographic technique in some experiments combined with direct recordings of changes in the regional blood volume Three different types of model experiments were performed in order to facilitate the analysis of the vascular events in the small intestine in a situation closely mimicking hemorrhagic shock The following observations were made during a 2 1/2 h hemorrhage amounting to 30–40 per cent of the calculated blood volume

a The intestinal resistance vessels constricted upon bleeding because of a sympathetically induced nervous vasoconstriction overruling the inherent myogenic properties of the resistance vessels to autoregulate blood flow During a prolonged period of hypovolemia accumulating local metabolites began to counteract the remote nervous control and blood flow resistance was eventually reduced to about half that of control Upon retransfusion resistance vessel tone remained below control

b The precapillary sphincters determining the surface area available for transcapillary fluid exchange relaxed upon bleeding probably due to their great sensitivity to local chemical dilating factors After retransfusion the tone of the precapillary sphincters was normalized

c The intestinal capacitance vessels containing the regional blood volume were rapidly constricted upon bleeding mainly due to a reflex nervous vasoconstriction A dilatation of the capacitance vessels was observed during a prolonged hemorrhagic hypovolemia caused by a local accumulation of dilating substances The increase of regional blood volume eventually amounted to about half the reduction seen upon bleeding

d An augmentation of tissue volume beyond that caused by the dilatation of the capacitance vessels was observed during the late half of hypovolemia in all probability induced by a derangement of the Starling equilibrium across the capillary wall This in turn was probably caused by an augmentation of the local colloid osmotic pressure in combination with an increased mean hydrostatic capillary pressure

2 Hemorrhagic lesions in the small intestinal mucosa localized in the villous tips were often seen after periods of reduced intestinal blood flow These mucosal lesions were microscopically described and compared to those seen in man and other experimental animals It was demonstrated that small amounts of oxygen supplied to the mucosa via an intraluminal perfusion of oxygenated saline could prevent the development of the lesions Intraluminal perfusion with nitrogenated saline on the other hand was unable to prevent them indicating that tissue hypoxia but not the luminal contents is the key factor in the pathogenesis of

the mucosal lesions

3 A new hypothesis concerning the pathogenesis of the mucosal lesions is presented This hypothesis is based on the existence of a counter current exchange mechanism in the intestinal mucosa According to this hypothesis tissue hypoxia is developing at the villous tips during arterial hypotension due to an increased extravascular short circuiting of oxygen between the two limbs of the countercurrent exchanger This in turn is probably induced by the decreased linear rate of blood flow in the hairpin vascular loops of the intestinal villi during hypotension

4 Substance(s) with a negative effect on cardiac performance was released from the small intestine during simulated shock conditions This blood borne material was in all probability produced in the small intestine during periods of reduced blood flow but was mainly released during the first posthypotensive min to judge from the rapid and pronounced decrease of cardiac performance observed during this period The cardio-depressant effect could be prevented if intestinal venous blood was collected during the first 4-5 min after the hypotension and substituted with blood from a healthy donor animal The true nature of the cardio-depressant material is not yet known A causal relationship between the hemorrhagic mucosal lesions and the intestinal release of the cardiotoxic material is suggested and it is proposed that the material may be products of the mucosal tissue destruction

ACKNOWLEDGEMENTS

I wish to convey my warmest thanks

to Professor Björn Folkow for the privilege of performing this experimental study at the Department of Physiology and for his support and invaluable constructive criticism

to Professor Ragnar Romanus head of the Department of Surgery II for the privilege of working in his clinic and for his valuable advice and constructive discussions

to Docent Ove Lundgren who introduced me to this field of re search for his valuable advice and co-operation Without his steadfast guidance this study would never have been completed

to Docent Christer Åhrén whose co-operation made the microscopic studies possible

to my colleagues in the Departments of Physiology and Surgery II including guest researcher Dr John Fara for valuable discussions

to the skilful technicians in the Department of Physiology especially Mrs Birgitta Vallander Mrs Dagny Johansson and Mrs Lisbeth Lindgren

to animal keeper Mr Gösta Magnusson

to photographer Mr Thomas Palm

to Miss Elsa Fredriksson for her valuable contribution to the atmosphere at the Department of Physiology

to Mrs Kerstin Lundgren and Mrs Sylvia Palmquist for skilful typing of the manuscripts

I also wish to express my gratitude to my wife Margareta for her patience and valuable support

This research has been sponsored by grants from the Swedish Medical Research Council (B73 14X 2855-03 and partly B73 14X 16-09B) from the Faculty of Medicine University of Göteborg from Svensk Kirurgisk Förening and from Svenska Sällskapet för Medicinsk Forskning

AB Hässle Göteborg Sweden generously covered part of the expenses for a technician and provided laboratory facilities for two control experiments

the mucosal lesions

3 A new hypothesis concerning the pathogenesis of the mucosal lesions is presented This hypothesis is based on the existence of a counter current exchange mechanism in the intestinal mucosa According to this hypothesis tissue hypoxia is developing at the villous tips during arterial hypotension due to an increased extravascular short circuiting of oxygen between the two limbs of the countercurrent exchanger This in turn is probably induced by the decreased linear rate of blood flow in the hairpin vascular loops of the intestinal villi during hypotension

4 Substance(s) with a negative effect on cardiac performance was re leased from the small intestine during simulated shock conditions This blood borne material was in all probability produced in the small in testine during periods of reduced blood flow but was mainly released during the first posthypotensive min to judge from the rapid and pro nounced decrease of cardiac performance observed during this period The cardio-depressant effect could be prevented if intestinal venous blood was collected during the first 4-5 min after the hypotension and substituted with blood from a healthy donor animal The true nature of the cardio-depressant material is not yet known A causal relationship between the hemorrhagic mucosal lesions and the intestinal release of the cardiotoxic material is suggested and it is proposed that the material may be products of the mucosal tissue destruction

BROWN R A C J CHIU H J SCOTT and F N GURD Ultrastructural changes in the canine ileal mucosal cell after mesenteric arterial occlusion Arch Surg 1970 101 290-297

BYGDEMAN S Vascular reactivity in cats during induced changes in the acid-base balance of the blood Acta physiol scand 1963 61 Suppl 222

CARTER D and A EINHEBER Intestinal ischemic shock in germ-free animals Surgery 1966 122 66-76

CELANDER O The range of control exercised by the sympathetico-adrenal system Acta physiol scand 1954 Suppl 116 1 132

CHALMERS J P P J KORNER and S W WHITE Effects of haemorrhage on the distribution of peripheral blood flow in the rabbit J Physiol (Lond) 1967 192 561 574

CHIEN S Role of the sympathetic nervous system in hemorrhage Physiol Rev 1967 47 214-288

CHIU C J A H McARDLE R BROWN H J SCOTT and F N GURD Intestinal mucosal lesion in low-flow states Arch Surg 1970 101 478-483

COBBOLD A B FOLKOW I KJELLMER and S MELLANDER Nervous and local chemical control of pre-capillary sphincters in skeletal muscle as measured by changes in filtration coefficient Acta physiol scand 1963 57 180-192

COOK B H E R WILSON and A E TAYLOR Intestinal fluid loss in hemorrhagic shock Amer J Physiol 1971 221 1494-1498

CULL T E M P SCIBETTA and E E SELKURT Arterial inflow into the mesenteric and hepatic vascular circuits during hemorrhagic shock Amer J Physiol 1956 185 365-371

DEDICHEN H Hemodynamic changes in experimental hemorrhagic shock Acta chir scand 1972 138 129-141

DUPUYTREN G Lecons orales de clinique chirurgicale V article XII des brulures Germer Baillière Paris 1839 pp 503-604

FARA J and U HAGLUND Release of serotoni from the small intestine following hypotension i the cat Europ Surg Res 1973 In press

FINE J The intestinal circulation in shock Gastroenterology 1967 52 454-458

FINE J E D FRANK H A RAVIN S H RUTENBERG and F B SCHWEINBURG The ba terial f cto i traumatic shock New Eng J Med 1959 260 214 220

FINK B R and A SCHOOLMAN Arte ial acid-base balance i unrestrained waking cats Proc Soc xp Biol (N Y) 1963 112 328 330

FOLKOW B Description of the myogenic hypothesis Circulat Res 1964 Suppl 1 14 15 279-285

FOLKOW B Regional adjustments of i testinal blood flow Gastroenterology 1967 52 423-432

FOLKOW B D H LEWIS O LUNDGREN S MELLANDER and I WALLENTIN The effect of graded asoconstri tor fibre timulation on the intestinal resistance and capacitance vessels Acta physiol scand 1964 a 61 445-457

FOLKOW B D H LEWIS O LUNDGREN S MELLANDER and I WALLENTIN The effect of sympathetic vasoconstrictor fibres on the distribution of capillary blood flow in the intestine Acta physiol scand 1964 b 61 458-466

FOLKOW B O LUNDGREN and I WALLENTIN Studies on the relationship between flow resistance capillary filtration coefficient and regional blood volume in the intestine of the cat Acta physiol scand 1963 57 270-283

FRIEDMAN J J Mesenteric circulation in hemorrhagic shock Circulat Res 1961 9 561 565

GELIN L-E Studies in anemia of injury Acta chir scand 1956 Suppl 210 1 130

GLENN T M and A M LEFER Significance of splanchnic proteases in the production of a toxic factor in hemorrhagic shock Circulat Res 1971 29 338 349

GLENN T M A M LEFER A C BEARDSLEY W W FERGUSON A M LOPEZ RASI T S SERATE J R MORRIS and S L WANGENSTEEN Circulatory responses to splanchnic lysosomal hydrolases in the dog Ann Surg 1972 176 120-127

GREENWAY C V and U S MURTHY Mesenteric vasoconstriction after endotoxin administration in cats pretreated with aspirin Brit J Pharmacol 1971 43 259 269

HAGLUND U O LUNDGREN and J SVANVIK On the pathogenesis of the mucosal lesions in shock Acta physiol scand 1973 87 49A 50A

HINSHAW L B L J GREENFIELD S E OWEN L T ARCHER and C A GUENTER Cardiac response to circulating factors in endotoxin shock Amer J Physiol 1972 222 1047 1053

HUGON J S and G BOUNOUS Intestinal lesions in low flow states electron microscopic study In Vascular disorders of the small intestine Ed S J Boley Butterworths London 1971

HULTEN L J LINDHAGEN and O LUNDGREN Blood flow in the parallel-coupled vascular sections of the human small intestine analyzed by ^{133}Xe wash-out technique Microvasc Res 1972 4 461

ISAKSSON O Influence of in vitro mechanical activity on protein synthesis and amino acid transport in perfused rat heart Thesis Göta-tryckeriet Göteborg 1972

JACOBSSON E D Are adrenergic overactivity and splanchnic vasoconstriction the prime pathophysiological events in shock? In The fundamental mechanisms of shock ed L B Hinshaw and B G Cox Plenum Publ Co N Y 1972

JOHNSON P C Origin localization and homeostatic significance of autoregulation in the intestine Circulat Res 1964 14-15 Suppl I 225-232

JOHNSON P C and E E SELKURT Intestinal weight changes in hemorrhagic shock Amer J Physiol 1958 193 135-143

KAMPP M O LUNDGREN and N J NILSSON Extravascular shunting of oxygen in the small intestine of the cat Acta physiol scand 1968 72 396-403

KEWENTER J The vagal control of the jejunal and ileal motility and blood flow Acta physiol scand 1965 65 Suppl 251

KLEMPERER P A PENNER and A I BERNHEIM The gastro-intestinal manifestations of shock Amer J Dig Dis 1940 7 410-414
KORNER P I Circulatory adaptations in hypoxia Physiol Rev 1959 39 687 730
KUIDA H Discussion of the intestinal circulation in shock Gastroenterology 1967 52 458-460
KUIDA H R P GILBERT L B HINSHAW J G BRUNSON and M B VISSCHER Species differences in effect of gramnegative endotoxin on circulation Amer J Physiol 1961 200 1197-1202
LEFER A M Role of a myocardial depressant factor in the pathogenesis of circulatory shock Fed Proc 1970 29 1836-1847
LEFER A M and T M GLENN Role of pancreas in the pathogenesis of circulatory shock In The fundamental mechanisms of shock ed L B Hinshaw and B G Cox Plenum Publ Co N Y 1972
LEWIS D H and S MELLANDER Competitive effects of sympathetic control and tissue metabolites on resistance and capacitance vessels and capillary filtration in skeletal muscle Acta physiol scand 1962 56 162 188
LILLEHEI R C The intestinal factor in irreversible hemorrhagic shock Surgery 1957 42 1043-1054
LILLEHEI R C R H DIETZMAN and S MOVSAS The visceral circulation in shock Gastroenterology 1967 52 468-471
LILLEHEI R C J K LONGERBEAM J H BLOCH and W G MANAX The nature of irreversible shock experimental and clinical observations Ann Surg 1964 a 160 682 708
LILLEHEI R C J K LONGERBEAM J H BLOCH and W G MANAX The modern treatment of shock based on physiologic pri ciples Clin pharmacol therapeut 1964 b 5 63-101
LINDEN R J The heart ventricular function Anaesthesia 1968 23 566-584
LONGERBEAM J K R C LILLEHEI W R SCOTT and J C ROSENBERG Visceral factors in shock J A M A 1962 181 878 883
LUNDGREN O Studies on blood flow distribution and countercurre t exchange i the small intestine Acta physiol scand 1967 Suppl 303
LUNDGREN O J LUNDVALL and S MELLANDER Range of sympathetic discharge and reflex vascular adj stments i skel tal muscle during hemorrhagic hypotension Acta physiol scand 1964 62 380-390
LUNDGREN O and J SVANVIK Mucosal haemodynam cs i the small intestine of the cat during reduced perfusion pressure Acta physiol scand 1973 i press
McNEILL J R R D STARK and C V GREENWAY Intestinal vasoconstri ti n after hemorrhage: roles of vasopressi and angiotensi Amer J Physiol 1970 219 1342 1347
MARSTON A The bowel i shock The rol of mesenteric arterial disease as a cause of death i the lderly Lancet 1962 a 2 365-370
MARSTON A The bowel i shock Lancet 1962 b 2 881
MARSTON A Acute mesenteric vascular occlusio in Vascular disorders of the small intestine Ed S J Boley Butterworths London 1971

MELLANDER S Comparative studies on the adrenergic neuro-hormonal control of resistance and capacitance blood vessels in the cat Acta physiol scand 1960 50 Suppl 176

MELLANDER S and D H LEWIS Effect of hemorrhagic shock on the reactivity of resistance and capacitance vessels and on capillary filtration transfer in cat skeletal muscle Circulat Res 1963 13 105–118

MILLIKEN J A NAHOR and J FINE A study of the factors involved in the development of peripheral vascular collapse following release of the occluded superior mesenteric artery Brit J Surg 1965 52 699 704

MING S -C Hemorrhagic necrosis of the gastrointestinal tract and its relation to cardiovascular status Circulation 1965 32 332 341

ÖBERG B Effects of cardiovascular reflexes on net capillary fluid transfer Acta physiol scand 1964 62 Suppl 229

ÖBERG B The relationships between active constriction and passive recoil of veins at various distending pressures Acta physiol scand 1967 71 233-247

OCHSENFAHRT H and D WINNE Der Einfluss der Durchblutung auf die Resorption von Arzneimitteln aus dem Jejunum der Ratte Arch Pharmak 1969 264 55-75

OWEN C A Diagnostic Radioisotopes Charles C Thomas Publ Springfield Ill 1959

PAPPENHEIMER J R Passage of molecules through capillary walls Physiol. Rev 1953 33 387–423

PENNER A and A I BERNHEIM Acute postoperative enterocolitis A study on the pathologic nature of shock Arch Pathol 1939 27 966–983

REYNELL P C P A MARKS C CHIDSEY and S E BRADLEY Changes in splanchnic blood volume and splanchnic blood flow in dogs after hemorrhage Clin Sci 1955 14 407–419

ROBINSON J W L J A ANTONIOLI and V MIRKOVITCH The intestinal response to ischemia Arch Pharmak exp path 1966 225 178 191

ROBINSON J W L and V MIRKOVITCH The recovery of function and microcirculation in small intestinal loops following ischemia Gut 1972 13 784 789

RÖTTGER P and M ORAN Histochemische und biochemische Untersuchungen zur Frage des Enzymgehaltes der Dünndarmschleimhaut nach temporärer Ischemie Verh dtsch Ges Path 1969 53 189 193

RUSH B F Irreversibility in the post transfusion phase of hemorrhagic shock In The fundamental mechanisms of shock ed L B Hinshaw and B G Cox Plenum Publ Co N Y 1972

SCHWEINBURG F B H A FRANK and J FINE Bacterial factor in experimental hemorrhagic shock: evidence for development of bacterial factor which accounts for irreversibility to transfusion in hemorrhagic shock in dog Amer J Physiol 1954 179 532 540

SCOTT I B R M DAUGHERTY and F I HADDY Effect of severe local hypoxemia on transcapillary water movement in dog forelimb Amer J Physiol 1967 212 847 851

SELKURT E E Mesenteric hemodynamics during hemorrhagic shock in the dog with functional absence of the liver Amer J Physiol 1958 193 599-604

SELKURT E E Intestinal ischemic shock and the protective role of the liver Amer J Physiol 1959 197 281 285

SELKURT E E Status of investigative aspects of hemorrhagic shock Fed Proc 1970 29 1832 1835

SELKURT E E R S ALEXANDER and M B PATTERSON The role of the mesenteric circulation in the irreversibility of hemorrhagic shock Amer J Physiol 1947 149 732 743

SELKURT E E and G A BRECHER Splanchnic hemodynamics and oxygen utilization during hemorrhagic shock in the dog Circulat Res 1956 9 693 704

SHAPIRO P B B BRONSTHER E D FRANK and J FINE Host resistance to hemorrhagic shock XI Role of deficient flow through intestine in development of irreversibility Proc Soc Exp Biol N Y 1958 97 372 376

SÖRENSEN F H and M VETNER Hemorrhagic mucosal necrosis of the gastrointestinal tract without vascular occlusion Acta chir scand 1969 135 439-448

SUTHERLAND N G G BOUNOUS and F N GURD Role of intestinal mucosal lysosomal enzymes in the pathogenesis of shock J Trauma 1968 8 350-377

SWAN K G R W BARTON and D G REYNOLDS Splanchnic blood flow in experimental shock In The fundemental mechanisms of shock ed L B Hinshaw and B G Cox Plenum Publ Co N Y 1972

SWAN H and A W NELSON Experimental lethal canine hemorrhage: ischemic abdominal viscera, absence of hemorrhagic gut or portal congestion J Surg Res 1971 11 620-628

SVANVIK J Mucosal blood circulation and its influence on passive absorbtion in the smal l intestine An experimental study on the cat Acta physiol scand 1973a Suppl 385

SVANVIK J Mucosal hemodynamics in the small intestine of the cat during regional sympathetic vasoconstrictor activation Acta physiol scand 1973b In press

WALLENTIN I Studies on intestinal circulation Acta physiol scand 1967 69 Suppl 279

WERLE J M R S COSBY and C J WIGGERS Observations on hemorrhagic hypotension and hemorrhagi shock Amer J Physiol 1942 136 401-420

WIGGERS C J The physi logy of shock Commonwealth Fund N Y 1950

WIGGERS C I D F OPDYKE and J R JOHNSON Portal pressure gradients under experimental conditions including hemorrhagic shock Amer J Phys ol 1946 146 192 206

WILLIAMS L F A M GOLDBERG B J POLANSKY and J J BYRNE Myocardi l eff cts of intestinal ischemia J Surg Res 1969a 9 319 323

WILLIAMS L F A M GOLDBERG B J POLANSKY and J J BYRNE Mycardial ffects of acute i testinal ischemia Surgery 1969b 66 138 144

ACTA

TOPOGRAPHY OF NEURON SYSTEMS IN AS REVEALED BY

LUND 1973

ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 388

FROM THE DEPARTMENT OF HISTOLOGY
UNIVERSITY OF LUND LUND SWEDEN

TOPOGRAPHY OF THE MONOAMINE NEURON SYSTEMS IN THE HUMAN BRAIN AS REVEALED IN FETUSES

BY

ANDERS NOBIN and ANDERS BJÖRKLUND

LUND 1973

INDEX OF ABBREVIATIONS

AC anterior commissure
ACC n. accumbens
ACP ascending catecholamine pathway
AIP ascending indolamine pathway
AP area postrema
CER cerebellum
CHOR choroid plexus
CL claustrum
CN caudate nucleus
CST corticospinal tract
DB n. of the diagonal band
DCP descending catecholamine pathway
DIP descending indolamine pathway
DM dorsomedial nucleus
F fornix
FLM medial longitudinal fasciculus
FR fasciculus retroflexus
GP globus pallidus
IC internal capsule
IF interpeduncular fossa
IN infundibular nucleus
INST bed n. of the stria terminalis
IOC inferior olivary complex
LC locus coeruleus
LN lentiform nucleus
LRN lateral reticular nucleus
MB mammillary body
ME median eminence
MFB medial forebrain bundle
MT mammillothalamic tract
N XII hypoglossal nerve nucleus
NR nucleus ruber
OB olfactory bulb
OC optic chiasm
OT olfactory tubercle
PA paraventricular nucleus
PCS superior cerebellar peduncle
PE periventricular nucleus
PF perifornical area
PH posterior hypothalamic area
PN paranigral nucleus
SC subcoeruleus area (level 1 C)
suprachiasmatic nucleus (level 1 L)
SE septum
SN substantia nigra, pars compacta (α β γ indicate the subgroups of Olszewski and Baxter [70])
SN substantia nigra, pars reticularis
STH subthalamic nucleus
V III third ventricle
V IV fourth ventricle
VM ventromedial nucleus
III oculomotor nerve

Printed in Sweden
Lund 1973 AB Grahns Boktryckeri

SUMMARY

The distribution of catecholamine (CA)-containing and indolamine (IA) containing structures in the human brain has been studied in 3—4 months old fetuses. At this stage of development, the CA and IA cell groups and major axon pathways were richly developed, whereas fluorescent varicose probably terminal, CA-containing fibres occurred abundantly only in certain well-developed regions, such as the hypothalamus, the basal ganglia, and the septal and olfactory regions. Apart from an area in the rostral pons no IA-containing varicose fibres were demonstrable.

A. The fluorescent cell bodies could be referred to seven larger cell formations, four composed of CA-containing cell bodies and three of IA-containing ones 1 A ventral and ventrolateral system of CA-containing cells in the medulla oblongata 2 CA-containing cells in the dorsal and dorsomedial regions of the medulla oblongata, partly within the area postrema 3 A large system of CA-containing cells in the dorsal and lateral regions of the pons, mainly within the locus coeruleus and the subcoeruleus area 4 An extensive system of CA-containing cells in ventral and medial regions of the mesencephalon and the rostral pons, partly within the pars compacta of the substantia nigra 5 and 6 Two formations of IA-containing cells in the midline raphe regions of the medulla oblongata and of the pons-mesencephalon 7 IA-containing cells in the lateral reticular formation in the caudal mesencephalon and the rostral pons. In addition, two small fluorescent cell groups were observed one composed of CA-containing cells in the infundibular (arcuate) nucleus of the hypothalamus, and one composed of IA-containing cells in the roof of the fourth ventricle.

B The developing non-terminal axons had a high amine content and were well visible in the fetuses. One major system of CA fibres could be traced from the region of the medulla oblongata up to the septal region. This system received fibres along its course from the CA cell bodies in the medulla pons, and mesencephalon, and probably gave rise to extensive terminal systems in the hypothalamus and in the olfactory and septal regions.

C. A nigro-striatal system of CA fibres could be established They ran from the groups of CA cell bodies in the substantia nigra region via the tegmental fields of Forel, the lateral hypothalamus, and the zona incerta into the internal capsule, towards the basal ganglia. Not only the caudate nucleus, the putamen, and the claustrum, but also the tissue of the developing globus pallidus were supplied with fluorescent varicose fibres, thus giving fluorescence histochemical evidence also for a nigropallidal, probably dopamine-containing projection in the human brain.

D In the infundibulum of the hypothalamus fluorescent cell bodies and terminals were demonstrated, corresponding to the tubero-infundibular dopamine neurons previously known to exist in laboratory mammals.

E. The topography of the monoamine neurons was compared with that of the rat brain. It is concluded that, although there are many differences with respect to details, the principles of organization of the monoamine neuron systems are notably similar in rat and man.

INTRODUCTION

The catecholamines, noradrenaline (NA) and dopamine (DA) and the indolamine, 5-hydroxytryptamine (5-HT serotonin) have wide-spread distributions in the central nervous system of all investigated mammals, including man [1, 4, 7, 8, 21, 26, 37, 70]. With the formaldehyde fluorescence method of Falck and Hillarp these monoamines have been demonstrated within cell bodies and terminals of central neurons of several laboratory mammals, including monkey [3, 22, 24, 25, 30, 46, 48], and the been established in detail in the rat brain [2, 18, 24, 25, 30, 78] (for reviews see refs. 31, 32, 56). Numerous experimental and pharmacological studies in animals have created great interest in the functional significance of the monoamine neurons in the CNS, not least with respect to mental, neurological and endocrine functions in man. Against this background it is remarkable how so little is known about the cellular localization of monoamines in the human brain. The major reason for this lack of knowledge is that this material offers special technical problems. Although the brain levels of catecholamines and serotonin decline rather slowly during the first few hours after death [7, 42, 64] their fluorescence histochemical visualization in central nervous tissues is possible only when the tissue is processed within the first 30—60 min after death [18, 71, 27]. Nyström *et al* [63] have circumvented this difficulty by using smear preparations of surgical biopsies of the cortex, and they were able to observe CA fluorescence in varicose fibres in human cerebral and cerebellar cortices in such preparations. Obviously, however, surgical biopsies cannot be used for more extensive neuro-anatomical studies.

In the present stydy the topography of central catecholamine and indolamine neuron systems has been investiged in fresh brains from 3—4 months old human fetuses. This material offered three major advantages. First, the fluorescence histochemical result was of high quality and in all respects comparable to that obtained with brains from laboratory animals, secondly, due to the high monoamine levels of the non-terminal axons of the developing neuron [61, 66] it proved possible to map not only monoamine-containing cell bodies and axon terminals, but also the major axon pathways in the human fetal brain, and thirdly, the material can be expected to be essentially non pathological and free from variations due to drug therapy.

MATERIAL AND METHODS

The material comprises brain tissue, pituitary glands, and pineal glands from human fetuses removed by caesarean section in connexion with legal abortion. The post mortem interval preceding freezing was usually 15—30 min, and in no case exceeded 1 h.

Fluorescence histochemistry. For the fluorescence histochemical study 32 fetuses were used; the crown — rump length (CRL) varied from 10 to 15 cm (gestation age about 3—4 months). Single observations were made in fetuses with a CRL up to 20 cm. The brain tissue and the pituitary and pineal gland were rapidly frozen in a propane propylene mixture cooled to the temperature

of liquid nitrogen. After the subsequent freeze-drying the specimens were processed for fluorescence microscopy according to the Falck—Hillarp method (for details, see ref 19) Special precautions were taken in the processing of the specimens in order to achieve an optimum visualization of adrenergic nerves (see ref 10)

Chemical analysis Brains from 3 fetuses with CRL of 10—15 cm were analysed fluorimetrically for the content of noradrenaline and dopamine according to the method of Bertler Carlsson Rosengren and Waldeck [4] as modified by Haggendal [22]

RESULTS AND COMMENTS

1 *General features of the monoamine neuron systems*

In the 3 to 4 month human fetuses, catecholamines (CA) and indolamines (IA) were detectable in cell bodies, in smooth or varicose non-terminal axon bundles, and in varicose, probably terminal, axon parts (*cf* ref 60) The whole brain catecholamine levels were determined in three fetuses they were found to be very low A mean of 101 ng of dopamine and 244 ng of noradrenaline was found per brain, corresponding to a concentration of 9 and 18 ng/g respectively None the less, abundant systems of fluorescent catecholamine-containing neurons were demonstrable.

Green fluorescent (CA-containing) and yellow fluorescent (IA-containing) cell bodies occurred in abundant systems in medulla oblongata, pons and mesencephalon, and some green fluorescent cell bodies occurred also in the hypothalamus. The cell body fluorescence was either rather diffuse covering also the nucleus, or more distinct and confined to the cytoplasm The fluorescent cell bodies could be referred to seven larger cell formation four CA-containing and three IA-containing 1 A ventral and ventro-lateral system of CA-containing cells in the medulla oblongata 2. CA-containing cells in the dorsal and dorsomedial regions of the medulla oblongata partly within the area postrema 3 A system of pontine CA-containing cells in the dorsal and lateral regions of the pons mainly within the locus coeruleus and the subcoeruleus area 4 An extensive system of CA cell in the ventral and medial regions of the mesencephalon (extending caudally into the rostral pons) mainly within the substantia nigra and the ventromedial tegmentum 5 and 6 Two formations of IA-containing cells in the midline raphe regions, one in the rostral part of the medulla oblongata, and another more extensive system, extending in the raphe region of the pons and the caudal mesencephalon 7 A less abundant group of IA-containing cells in the lateral reticular formation, in the caudal mesencephalon and the rostral pons In addition, a small group of CA-containing cells was observed in the infundibular nucleus of the hypothalamus, and a small group of IA-containing cell occurred in the roof of the fourth ventricle No fluorescent cell bodies were detected in other diencephalic regions or in telencephalon

The monoamine axon bundles had a notably high fluorescence and could thus be traced for long distances through the lower brain stem and the hypothalamus up to the basal ganglia and the septal region. One major system of CA fibres could be followed from the region of the lower medulla oblongata through pons and mesencephalon at the level of the red nucleus, it joined the medial forebrain bundle (MFB) Within the MFB, one system of CA fibres could be further traced up to the septal region. Along the course of this long, probably ascending, fibre system, it received fibres from the CA cell systems in medulla, pons and mesencephalon.

A second large system of CA fibres ran from the region of the mesencephalic CA cell bodies in the pars compacta of the substantia nigra and in the ventromedial tegmentum through the tegmental fields of Forel, the lateral hypothalamus and the zona incerta into the internal capsule, towards the basal ganglia. This system is probably identical with the so-called nigrostriatal dopaminergic pathways (see below)

In the medulla oblongata, two small, probably descending, CA-containing fibre bundles were observed in the ventral and dorsolateral regions. These bundles could be traced back to CA cells in the ventral and the dorsal medulla cell systems, respectively

Two IA-containing axon bundles were also visible one prominent, probably ascending bundle near the midline in the pons and the caudal mesencephalon and one more sparse, probably descending, bundle in the medulla oblongata close to the ventral surface.

Fluorescent varicose, probably terminal axon parts were detected to a more limited extent and only in certain regions. The most abundant CA terminal systems had developed in the basal ganglia and the olfactory and septal regions. CA terminals were also observed in parts of the hypothalamo-hypophyseal region and in the pineal, whereas remaining brain parts, including the lower brain stem, cerebellum, thalamus, and cortical structures, had no or only few fluorescent varicose fibres. Except for a restricted periventricular region in the rostral pons, no yellow fluorescent varicose IA terminals could with certainty be demonstrated in the studied fetuses.

The distribution of the histochemically detectable CA and IA neuron systems in the brain of 3—4 months old human fetuses is illustrated in Figs. 1 A—M. The distribution of the monoamine neuron systems in the human, as revealed in the fetal material is often similar to, but sometimes clearly different from, that in the rat, the mammalian species whose anatomy of the monoamine neuron systems is best known. In the subsequent paragraphs, the present observations will therefore be viewed particulary in comparison with previous observations in the rat brain

2. *Medulla oblongata (Figs 1 A and B)*

CA neurons The ventrally and ventrolaterally situated cell system was locali zed lateral to the developing inferior olivary complex (IOC) and extended caudally to the transition zone to the spinal cord. Most of the cells occurred

ventromedial to the lateral reticular nucleus (LRN) close to the ventral surface (Fig ? A) but at the level of the caudal half of the inferior olivary complex, they were also found scattered within the lateral reticular nucleus and extended scattered dorsomedially up to the ascending CA fibre bundle (ACP) The group consisted of small angular cells with a fluorescence that usually also covered the nucleus. Fluorescent, predominantly varicose fibres could be traced from the cells two directions (Figs. 1 A and ? A) dorsomedially up to the ascending CA bundle (arrows in Fig ? A) and ventrolaterally towards the probably descending bundle (DCP) close to the ventral surface. This cell group corresponds well to the A1 group of Dahlström and Fuxe [21] in the rat brain, and it seems likely that, similar to the arrangement in the rat [25, 32, 66, 73] the A1 cells could give rise to both descending fibres to innervate the spinal cord and ascending fibres to innervate higher brain centres.

In the dorsal and dorsomedial system of CA cells in the medulla, three separate cell groups could be distinguished One group of small, rounded, closely packed cells occurred in the developing area postrema (Fig B) Most of the cells had a less distinct appearance than the other medulla cells, the fluorescence was rather weak and covered also the nucleus, and they were generally without fluorescent processes. These area postrema cells had thus an appearance very similar to the adrenergic neuroblasts described by Olson and Seiger [64] in early stages of development in the rat brain (9th to 13th day of gestation) This suggests that, in the 3 to 4 month human fetuses, the CA cells of the area postrema are in a more premature stage of development than e.g. other medulla CA neurons. Interestingly in the lateral and caudal parts of the area postrema, the fluorescent cells were more strongly fluorescent and were markedly elongated and rows of such cells could be traced from the caudal part in ventral and ventrolateral direction and from the lateral parts in lateral direction towards he more mature looking CA cells. This could signify a developmental connexion between the neuroblast like cells of the area postrema and the more mature CA neurons in the medulla. Smooth green-fluorescent fibres could be traced in lateral direction from the area postrema. The occurrence of CA cells in the developing human area postrema agrees with the occurrence of such cells in the area postrema of a number of other mammals, including monkey [23]

The second group of CA neurons in the dorsal region of the medulla oblongata comprised larger angular fluorescent cells that were found scattered dorsal and lateral to the nucleus of the hypoglossal nerve (N XII) whose cells were readily observable in the fluorescence microscope These CA cells had a cytoplasmic fluorescence of moderate intensity they were most often spindle shaped and usually equipped with two fluorescence processes. This group corresponds to the A2 group in the rat brain (*cf* ref 24) In the rat, as in the human fetus (Fig 1 A) this cell group lies close to the ascending CA fibre bundle it has been suggested that, in the rat these cells contribute to the bundle [73]

A third, small group of dorsal CA cells occurred lateral to the area postrema.

Fig. 1 Frontal sections of the human fetal brain (Pages 9—12)

The localization of monoamine-containing cell bodies, axons and terminals are schematically represented in frontal sections of the fetal brain. The line drawings express the summarized impressions from many fetuses with a CRL varying from 10—20 cm (gestation age 3—4 months). The sections are cut in right angles to the longitudinal axis of each brain region. The levels A—M are taken in caudal-rostral direction. A and B represent two levels of the medulla oblongata. C and D two levels of the pons. E, F and G three levels of the mesencephalon. H, I, K, L and M represent five levels of the diencephalon and the basal telencephalon. For abbreviations see Index (p. 2). For further anatomical details, see Hochstetter [illegible]

Indications used



CA-containing cell bodies

IA-containing cell bodies

Longitudinally represented CA-containing axons

Longitudinally represented IA-containing axons

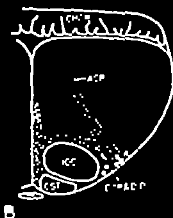
Transversely represented CA-containing axons or varicose terminals.

Transversely represented IA-containing axons or varicose terminals.

The cells were fairly large, moderately fluorescent with processes forming a laterally and caudally oriented fibre bundle that also seemed to receive fibres from the area postrema cells. This CA cell group does not seem to have any equivalent in the rat brain.

IA neurons Yellow fluorescent cell bodies were present in large numbers in the raphe region along the cranial half of the developing inferior olivary complex up to the level of the facial nerve. The cells extended laterally on the dorsal and dorsolateral surface of the inferior olivary complex caudally the IA cells intermingled with the green-fluorescent CA cells in the ventral medulla cell group (the A1 group). The area of distribution of the yellow fluorescent cells appeared to correspond to nucleus raphe pallidus, nucleus gigantocellularis, and nucleus paragigantocellularis lateralis in the adult brain (*cf* ref 70). The cells were generally weakly fluorescent, rounded or spindle

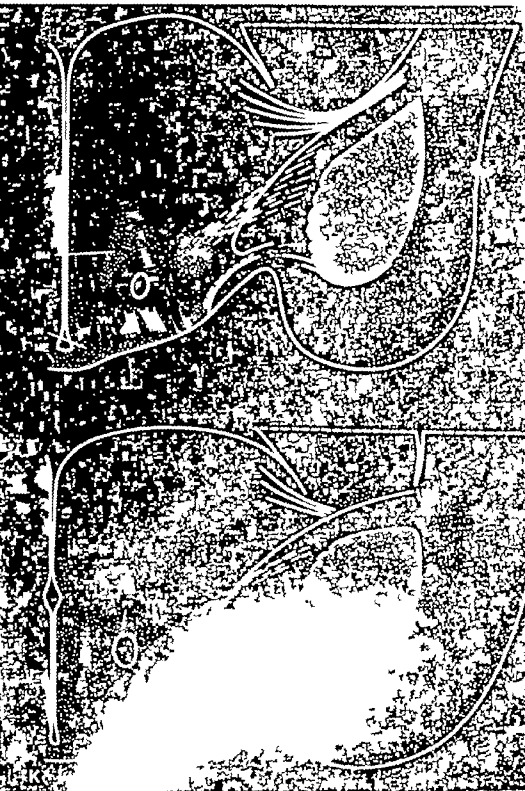


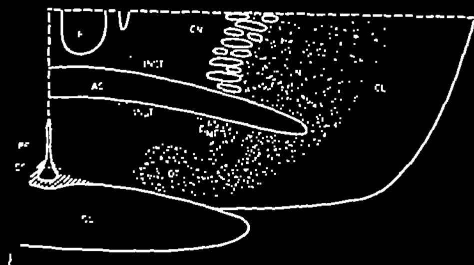






shaped with the long axis predominantly oriented dorsoventrally along the midline These cells correspond to the B1 B2, and B3 cell groups of Dahlström and Fuxe [34] in the rat brain, but in the human fetuses there was no obvious such grouping of the cells.







Smooth yellow fluorescent fibres were observed medially among the IA cells of the raphe region with a dorsoventral direction and laterally from the region of the laterally situated IA cells in a ventrolateral direction up to the surface intermingling with the similary oriented green fluorescent CA fibres (Figs. 1 A and B) This fibre arrangement is very similar to that observed in the rat [25, 29] It thu seems probable that also in the human brain at least part of

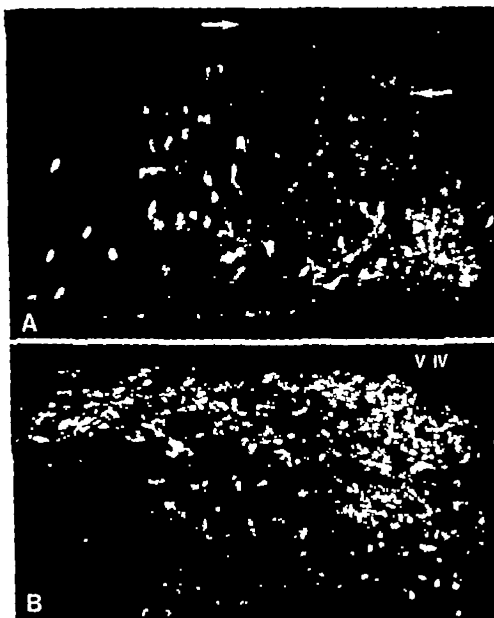


Fig. 2. Frontal section through medulla oblongata approximately [illegible] level represented by Fig. 1 A.

A. Green-fluorescent nerve cell bodies and fine, varicose green fluorescent fibres [illegible] observed close [illegible] the [illegible] surface [illegible] with [illegible] dorsomedial direction are indicated by arrows (X 160)

B. Green-fluorescent cell bod[illegible] [illegible] some of the cell bodies, mainly in the lateral portion (left) [illegible] fluorescence while others have weaker fluorescence and a [illegible] (V IV = fourth [illegible] entricle) (X 160)

the B1—B3 cells form a descending bulbo-spinal indolamine system forming a bundle at the ventral surface of the medulla oblongata (DIP in Figs. 1 A and B)

3 *Pons (Figs 1 C and D)*

CA neurons An abundant system of fairly large angular green-fluorescent cells occurred in the dorsal and lateral regions of the pons, mainly within the locus coeruleus and the so-called subcoerules area (Figs. 1 C and D Fig 3 A see refs. 70 74) These are the nuclei where pigmentation will occur later in development. Two main groups of cells could be distinguished within this region One elongated group consisted of rather densely packed cells and was probably identical with the locus coeruleus. From the locus coeruleus, a band of CA cells extended first laterally (Fig. 1 C) and then caudally and dorsally along the superior cerebellar peduncle into the roof of the fourth ventricle. These cells appear to be identical with the nucleus pigmentosus tegmentocerebellaris of Riley [73] another nucleus that become pigmented. The ascending CA bundle was situated immediately ventrolateral to the locus coeruleus cell group and there was a continuous flow of fibres from the locus coeruleus cells into this bundle. In addition, there was a substantial flow of fibres in dorsal direction from the locus coeruleus (and possibly also the ascending bundle) turning dorsomedially along the superior cerebellar peduncle towards the cerebellum. The second CA cell group occurred just ventral and ventrolateral to the ascending CA bundle (Fig 3 B and 4 A) most probably within the subcoeruleus area [70] Posteriorly these cells became more scattered extending into the area medial to the facial nerve. From these cells, fibres could be traced towards the ascending CA bundle. A flow of CA fibres with a lateral direction was also observed the fibres turning dorsally and ventrally along the lateral surface (Fig 1 C)

Also in the rat brain the locus coeruleus (group A6) with its caudal and dorsal extension (A4) and the subcoeruleus area (A7) contain CA (probably NA-containing) nerve cells [24, 83] In addition, a more caudally and ventrolaterally situated cell group is distinguished in the rat (group A5 *cf* ref 77) If equivalent cells occurred in the human fetus, they were not clearly separated from the subcoeruleus cell system. It has been demonstrated in the rat that the coeruleus and the subcoeruleus cell groups innervate diencephalic and telencephalic regions [2, 12, 78] and that the dorsolateral part of the locus coeruleus projects also to cerebellum [66] The arrangement of the fluorescent

---

Fig 3 Frontal section through pons at a level similar to the one in Fig. 1 C. A. Rather densely packed fluorescent cell bodies and varicose fibres within the locus coeruleus. (X 150)

B Higher magnification of fluorescent cell bodies in the subcoeruleus area. Fluorescent processes and non-fluorescent dark nuclei are clearly seen. Fluorescent varicose fibres are distributed between the cell bodies. (X 220)

A

B

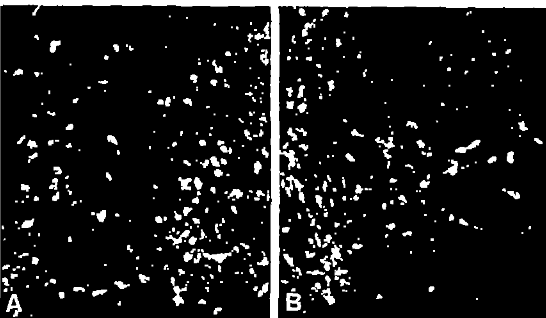


Fig. 4 Frontal section through the pons at a level close to the one in Fig. 1 D. Yellow fluorescent IA-containing cell bodies are seen in the raphe region of the rostral pons. The cells occur rather densely packed close to the midline (A ×120) and scattered in more lateral position (B ×120). The IA-containing cells are difficult to photograph due to their rapid photodecomposition.

axons in the human fetus is consistent with such an organization of the coeruleus and subcoeruleus CA neurons also in the human brain.

In the most rostral portion of the pons, two further groups of CA-containing cells occurred (Fig. 1 D). One within the central grey medial to the most rostral part of the locus coeruleus, and one ventrally in the area dorsal and dorsomedial to the medial lemniscus. The latter cell system continued rostrally into the mesencephalon, merging with the CA cell system in the substantia nigra (Figs. 1 E and F, see below). These ventral cells probably correspond to the A8 group of Dahlström and Fuxe [24] in the rat brain, although the cells extended more caudally in the human, whereas the medial group of cells within the central grey appears to have no equivalent in the rat brain.

IA neurons The yellow fluorescent IA-containing cells could be referred to one medial (Fig. 4 A) and one lateral (Fig. 4 B) system of cells both of which continued rostrally into the caudal mesencephalon. The medial system was the most extensive: it was localized in the central grey and the raphe region of the rostral pons. The cells were weakly fluorescent, rounded or spindle shaped with the longitudinal axis oriented dorsoventrally along the raphe.

Smooth, yellow fluorescent fibres, apparently forming ascending axon bundles, were observed in two locations (Figs. 1 C and D). First, in the central grey near the ependyma of the fourth ventricle and the aqueduct, and second, in a position between the medial raphe cells and the lateral cell

system (AIP) This latter bundle could be traced rostrally through the decussation of the superior cerebellar peduncles (Fig 1 E)

The medial pontine IA cells are equivalent to the B5 and B6 groups in the rat brain. The lateral cells should correspond to the B9 group and the laterally situated B8 cells in the rat, [14, 34] but in the human fetus these cell reach further caudally and seem to be more distinctly separated from the medial cell system than in the rat.

4 *Mesencephalon (Figs 1 F F and G)*

CA neurons A large number of weakly to moderately green wluorescent cells were present within the substantia nigra and the ventromedial tegmentum. The cells varied in size many of them having an indistinct outline with the fluorescence covering also the nucleus. In places, groups of cells with fluorescent processes outlined vessels, forming rows or a sheath of cells with broad processes oriented along the surface of the vessel (Fig 4 B) These cell sometimes had a conspicuously immature appearance and it seems conceivable that such cells could be young neurons or neuroblasts migrating along vessels.

The main portion of the mesencephalic CA cells was localized in the pars compacta of the substantia nigra (Figs. 5 C and D) The pars reticularis of the substantia nigra appeared to lack fluorescent cells The pars compacta cells could be referred to four major groups. The most densely packed occurred in the paranigral nucleus (Fig 5 C) (terminology according to Olszewski and Baxter [70]) These cells had a characteristic fluorescence morphology the fluorescence being rather diffuse and the outline of the cells being quite indistinct. The largest group occurred in the ventral part of the region, bordering the pars reticularis. These cells were arranged in aggregates or clusters (Fig. 5 D) corresponding to the α-subgroup of Olszewski and Baxter [70] Dorsal and dorsolateral to these aggregated cells, there occurred more scattered fluorescent cells corresponding to the β subgroup which was caudally continuous with the ventrally located CA cell group of the rostral pons (probably corresponding to the A8 group in the rat brain see above) The fourth subgroup formed a layer of fluorescent cells outlining the ventral surface of the nucleus ruber (Fig 6 A) corresponding to the γ-subgroup Medially in the substantia nigra, a flow of smooth green-fluorescent fibres could be followed from the CA cells dorsomedially and then turning rostrally and laterally around the nucleus ruber to run into the ascending MFB system of fibres. Here they merged with the CA fibre system ascending from the medulla oblongata and pons. Another flow of fibres ran laterally and rostrally through the region of the tegmental fields of Forel and the zone incerta, assembling into a compact bundle just dorsal to the subthalamic nucleus, probably within the fasciculus lenticularis, that continued into the internal capsule (Figs. 6 A and B) At the rostral portion of the nucle ruber (Figs. 1 G and 6 A) the ascending CA fibre systems had a capsule-like arrangement around the nucleus This arrangement was formed by the ascending fibres from the medulla-pons and from the mesencephalon assembling just rostral to the nucleus ruber (Fig 1 H)

Besides in the substantia nigra mesencephalic CA cells were found also in the ventromedial tegmentum, from the ventral brain surface up to the aqueduct. They occured near the midline from the rostral end of the inter peduncular nucleus up to the region medial to the fasciculus retroflexus (Figs. 1 F and G) Ventrally there was no distinct borderline towards the CA cells in the substantia nigra.

Principally the arrangement of the mesencephalic CA cells in the human fetus is similar to that of the rat brain (*cf* ref 24) However in the rat. the largest CA cell group is that of the ventromedial tegmentum (group A 10) whereas in the human fetus, the substantia nigra cell group (group A9 in the rat) is by far the most predominant.

IA neurons The IA-containing cell bodies of the mesencephalon were con fined to its caudal part (Fig 1 E) appearing as rostral extensions of the pontine IA cell systems. The medial system of cells became separated into a larger dorsal group situated around and dorsal to the medial longitudinal fasciculus (FLM) and a smaller midline group localized just ventral to the decussation of the superior cerebellar peduncles (Fig 4 B) Some scattered yellow fluor escent cells were also found between these two groups. These two mesencephalic IA cell groups appear to be comparable to the B7 and B8 groups of the rat brain [14, 24] In rat, the mesencephalic IA neurons innervate diencephalic and telencephalic regions mainly via the ascending medial forebrain bundle [20, 26, 31, 43]

5 *Diencephalon (Figs 1 H I K and L)*

In regions rostral to the mesencephalon only green-fluorescent CA-containing structures were demonstrable. One small group of diencephalic CA cell bodies was detected in the infundibular (arcuate) nucleus at the level of the posterior median eminence (Figs. 1 H and 7 B) These cells were notably small, weakly fluorescent, and immature looking Green-fluorescent varicose fibres occurred in several hypothalamic and hypophyseal areas, whereas in the thalamus, CA

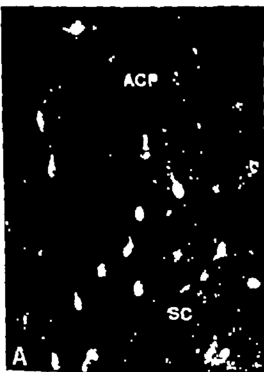
Fig 5 A Frontal section through pons at approximately the level indicated in Fig 1 C. CA cell bodies are seen ventral to the ascending catecholamine pathway (ACP) in the subcoeruleus area. In the ACP a large number of transversely cut fluorescent axons can be observed. (X 150)

B. Frontal section through mesencephalon at a level close to the one indicated in Fig 1 F A typical grouping of CA cells with broad fluorescent processes are seen outlining a tangentially sectioned vessel in the pars compacta of the substantia nigra (see text) (X 180)

C. Frontal section level corresponding to Fig. 1 G Within the paranigral nucleus small densely packed CA cells occur exhibiting a diffuse fluorescence and an indistinct outline (X 140)

D Frontal section level corresponding to Fig 1 G. CA cells within the pars compacta *of the substantia nigra. These cells correspond to the α-group in Fig. 1 G* (X 140)

For abbreviations, see Index (p. 2)


ACP
SC
A


B


IF
C


SNr
D

fluorescence was observed only in some scattered varicose fibres in ventricle near regions. A variable density of fluorescent varicose fibres was detected in the subthalamic nucleus. The pineal was supplied with CA fibres organized into thick bundles at the surface or within interlobular septa. These fibres had the appearance of peripheral sympathetic fibres (see below)

A dense CA fibre supply was found in the posterior hypothalamic area (the nomenclature follows essentially that of Kuhlenbeck [41] and Nauta and Haymaker [illegible]) the dorsomedial nucleus (Fig 8) the perifornical area, the mamillary body the tuberal nucleus, the periventricular nucleus (Fig. 9 C) the infundibular nucleus, the paraventricular nucleus (Fig 9 D) the supra chiasmatic nucleus, and in the median eminence- pituitary region (Figs. 7 A and C) Some scattered fibres were also observed in the dorsal, lateral and preoptic hypothalamic areas, and in the ventromedial nucleus, where they appeared as parallely arranged fibres traversing the nucleus in ventral and ventrolateral directions (Fig 1 I) In the rostral infundibulum (Fig 1 K) two systems of fibres extended between the infundibulum and the region of the MFB in a manner suggestive of a commissural arrangement.

In the median eminence-pituitary region, varicose CA fibres were demonstrated both in the internal and external layers of the developing median eminence (Fig 7 A) in the stalk, and in the neural lobe (Fig 7 C) In the external layer of the median eminence, some of the fibres were oriented perpendicular to the surface.

The ascending CA fibre system could be traced within the lateral hypothalamus in a position corresponding to the MFB and the medial zona incerta (see Fig 6 B) All along its course, a massive flow of fibres was seen to leave this system laterally to run through the zona incerta into the internal capsule towards the striatum. At the level of the rostral infundibulum (Fig 1 K) the lateral fibre system was divided into two components one dorsal running into the internal capsule, and one ventral running into the dorsal part of the developing globus pallidus. These fibre systems running towards the basal ganglia are probably components of the nigro-striatal dopamine fibre system that is the source of the dopaminergic innervation of the striatum (see refs. 2, 4 34 57 78) The supply of CA fibres in the striatum is further dealt with below

Rostrally the MFB system of CA fibres could be followed into the preoptic region in a position just ventral to the anterior commissure (Fig 1 L) Further

---

Fig 6 A. Frontal section through the mesencephalon t the level of the most rostral portion of the nucleus ruber (*cf* Fig 1 G) Photomontage showing brightly fluorescent preterminal axons around the nucleus ruber (NR) (Lateral is to the left of the figure) This fibre arrangement is formed by the CA fibres from the medulla-pons and the mesencephalon forming the ascending nigro-striatal and MFB fibre systems. The continuation of the laterally directed fibres (probably part of the nigro-striatal pathway) is seen t a mor rostral level in Fig B. A few green fluorescent cell bodies are seen medial to the fasciculus retroflexus (FR) and also in the ostral part of the pars compacta of the substantia igra (SN) (β in Fig 1 G) (X 90)



B. Frontal section through the caudal diencephalon at level corresponding to Fig. 1 H showing the nigro-striatal CA (probably dopamine) containing fibre system at level just rostral to A. The dorsal part of MFB is seen to the right in the picture and the internal capsule (IC) to the left. Some fluorescent varicose CA fibres can be seen in the subthalamic nucleus (STH) (X 90)

rostrally the fibres ran into the extensive systems of CA terminals in this region (see below)

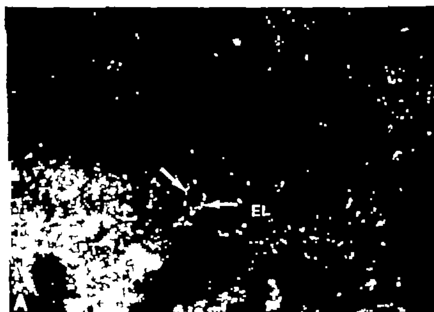
A comparison with the distribution of the fully developed CA innervations in adult rodents makes it clear that CA-containing terminal fibre systems in the human diencephalon are only partially developed in the 3—4 month fetuses. Most conspicuously only few CA fibres restricted to periventricular areas were demonstrable in the thalamus. None the less, there are definite similarities in the distribution of hypothalamic and hypophyseal CA fibres between the human fetus and rodents. These similarities are even more evident when comparing the 3—4 month human fetus with the situation in developing mice or rats during the early neonatal period [9, 22, 49, 50, 66, 70] At this stage of development, the thalamus is thus sparsely supplied with fluorescent varicose fibres, whereas the patterning but not the density of the hypothalamic CA innervations is largely comparable to that of the adult. In the median eminence and the pituitary neural lobe of the human fetus, the distribution and pattern of varicose CA-containing fibres are very similar to those of the tubero-hypophyseal CA neuron systems in the 1 week old rat or mouse. In rat, the dopaminergic components of the median eminence-pituitary innervations most probably originate in the arcuate nuclei [11, 14, 41, 47] The occurrence of CA containing cell bodies in the infundibular (arcuate) nucleus of the human fetus thus probably signifies the existence of such tubero-hypophyseal dopamine neurons also in the human brain, giving rise to the CA terminals in the median eminence and the pituitary neural lobe. In rat and mouse, there is, in addition, a direct innervation of the pars intermedia by CA fibres originating in the arcuate nuclei [19] this innervation was lacking in the human. The rostral and dorsal hypothalamic cell groups (A14 and A13) present in the rat diencephalon [18, 22] were not detectable in the human fetuses.

The hypothalamic areas that were richly supplied with CA fibres in the human fetus have such an innervation also in rat and mouse [9, 22, 30, 50, 64] These areas include the posterior hypothalamic area, the periformical area, and the dorsomedial, paraventricular and infundibular (arcuate) nuclei. It is notable, however that the suprachiasmatic and the subthalamic nuclei which had a significant CA fibre supply in the human fetus, have a poor CA innervation in rat. In rat, the suprachiasmatic nucleus has a dense indolamine (probably 5-hydroxytryptamine-containing) innervation [19, 30] that was not detectable in the present material

---

Fig 7 A Frontal section through the median eminence at level corresponding to 1 H Scattered varicose green-fluorescent fibres can be seen mainly within the external layer (EL) Note the perpendicula arranged fluorescent fibres in the external layer (arrows) Ventrally the pars tuberalis (PT) of the pituitary can be observed. (X 40)

B. Frontal section through th basal hypothalamus t the level of the posterior median eminence (*cf* Fig 1 H) The picture is turned to that ventral is to the right. In the infundibular ucleus lose t the entral surface few weakly fluorescent cell bodies are seen (rrows) (X 160)







C The neural lobe of the huma[illegible] fetal pituitary showing [illegible] network of delic[illegible] te [illegible] green-fluorescent fibres These fibres are seen in the neural lobe parenchyma without [illegible]y [illegible] rela[illegible]ion to blood vessels (X 180)



Fig 8 Frontal section through the diencephalon at level corresponding to Fig 1 L Photomontage showing the rather dense supply of CA-containing varicose fibres in the dorsomedial nucleus (DM) A few green-fluorescent fibres can also be seen in periventricular reas (VIII = third ventricle) and in the entromedial nucleus (VM) (× 80)

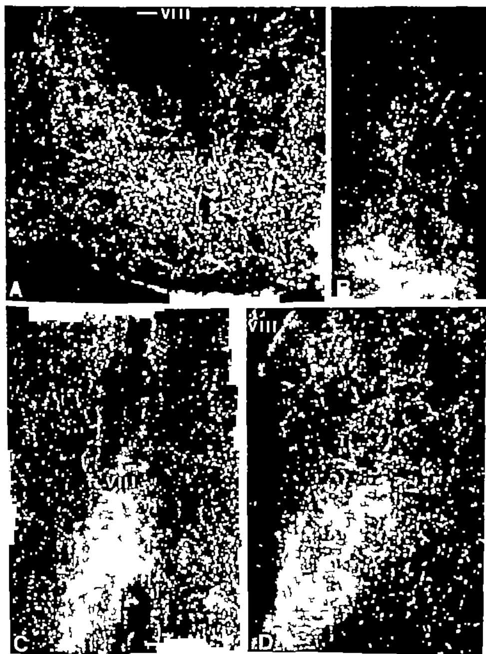
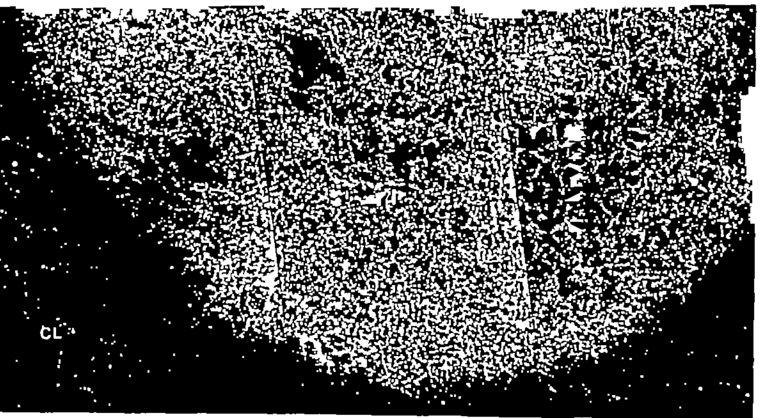


Fig. 9. Frontal sections through hypothalamus and cortex at level corresponding to Fig. 1 K. A supply of fluorescent varicose CA-containing fibres is demonstrated in the rostral infundibulum (A ×180) in the periventricular nucleus (C ×180) and in the paraventricular nucleus (D ×180). Some scattered CA fibres are also seen in the tissue of the developing cortex (B ×140). VIII = third ventricle.



F g 10 F ontal section at the level of caudal neostriatum (*cf* Fig 1 K) Photomontage of the entral part of the putamen showing the ery dense CA innervation of this ucleus. Some CA fibres are also present in the claustrum (CL) (× 140)

6. *Neostriatum and globus pallidus (Figs. 11 K, L and M)*

Among the structures of the basal ganglia (the nomenclature used is that of Metter [66]) the globus pallidus is the least developed in the fetuses used in the present study [66]. Thus, within the lentiform nucleus, the putamen by far predominates, and in the fluorescence microscope, the developing globus pallidus was identified with certainty only at the level of the rostral infundibulum (Fig. 1 K). Green fluorescent varicose fibres occurred throughout the entire lentiform nucleus and in the region of the rostral caudate nucleus bordering to the internal capsule (Figs. 10 and 11). Some scattered varicose fibres occurred also in the claustrum, which is part of the neostriatum complex [66]. Together with the olfactory region (see below), the neostriatum exhibited the densest CA fibre supply in the studied specimens. The predominating type of fluorescent fibre in the neostriatum had quite delicate and regular fluorescent varicosities. Frequently, such delicate beaded fibres were seen to end with a bulbous swelling (Fig. 11 E), probably identical with the growth cones described in the developing rabbit neostriatum [77]. Within the putamen, there occurred areas of a denser aggregation of the fluorescent fibres appearing as more strongly fluorescent islands (Fig. 11 B). Such fluorescent islands, which were most conspicuous in the younger fetuses analysed, are characteristic for the dopaminergic innervation in the developing neostriatum of rat and rabbit [50, 67, 77]. This patterning of the fluorescent fibres might reflect an organizational principle of the nigro-striatal dopamine system common for all mammals, including man.

Besides the delicate fluorescent fibres, there occurred a second, coarser type of fibre in the putamen (Fig. 11 F) and the caudate nucleus. They were considerably fewer than the delicate fibres and appeared to be randomly scattered among the delicate ones. The coarser fibres had a regular varicose appearance resembling the CA (probably noradrenaline-containing) fibres present, *e.g.* in the hypothalamus. A similar coarse fibre type has been demonstrated also in the neostriatum of the adult rat by means of the glyoxylic acid fluorescence method [61] and it seems feasible that they could represent the storage sites for the striatal noradrenaline.

The fluorescent fibres in the globus pallidus (Fig. 11 A) had a varicose appearance similar to that of the neostriatal fibres. But their varicosities were somewhat larger than the delicate ones in the neostriatum and resembled more the fibres detected in the subthalamic nucleus. The fluorescent fibres appeared evenly distributed throughout the tissue of the developing globus pallidus.

The presence of a nigro-neostriatal dopaminergic neuron system is well established in laboratory mammals, including the monkey [3, 4, 23, 67, 78]. This system projects abundantly to the neostriatum, but in rat, the globus pallidus and the claustrum appear to be devoid of catecholamine terminals [20, 22]. In the adult human brain dopamine (and in much lower concentrations also noradrenaline) occurs in both the neostriatum and the globus pallidus [5, 26, 29, 67, 78]. The present fluorescence microscopical findings demonstrate that these

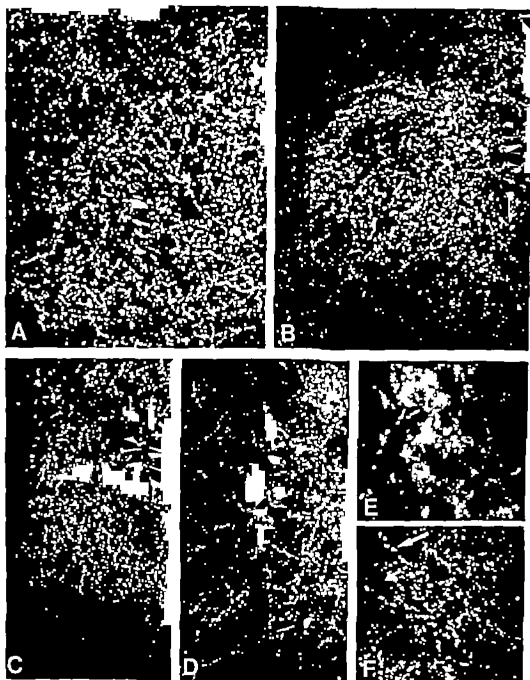


Fig. 11 A. Frontal section through globus pallidus (at level 1 K). The picture shows the dense supply of varicose CA fibres in this nucleus. The varicosities are somewhat larger than the delicate ones in the neostriatum (*cf.* Fig. 10 and Figs. 11 B and C). (× 190)
B. Frontal section. A dense aggregation of fluorescent varicose CA fibres appearing as more strongly fluorescent island in the putamen, characteristic of the DA-innervation in the developing neostriatum. (× 150)

catecholamines are localized in varicose fibres, probably nerve terminals, indicative of a dopaminergic (and probably also noradrenergic) innervation not only of the neostriatum but also of the globus pallidus in the human brain (cf Discussion)

7 Olfactory tubercle and septal region (Figs 1 L and M)

In the fluorescence microscope the CA fibre system in the rostral neostriatum continued ventrally into the olfactory tubercle (terminology according to Nauta and Haymaker [89]) The fluorescent varicose fibres in this region had the same general appearance as those in the neostriatum (see above Figs. 12 and 13 A) Ventrally and medially the fibres were aggregated into fluorescent islands, most probably identical to some of the so-called islands of Calleja. Both the nucleus accumbens (Fig 12) and the bed nucleus of the stria terminalis (Fig 14 A) were rather densely innervated by delicate varicose fluorescent fibres of the same type as in the neostriatum and the olfactory tubercle The fibre systems of these two nuclei were directly continuous, and rostral to the anterior commissure, there was no clear separation towards the fibre systems of the olfactory tubercle (ventrally) and the caudate nucleus (dorsolaterally) (Fig. 1 M)

Scattered, predominantly parallely arranged, varicose fluorescent fibres were present within the nucleus of the diagonal band (Fig 13 D) and the fibres continued dorsally into the medial part of the septum, where some scattered CA fibres occurred. A denser fluorescent varicose CA fibre supply was detected in the lateral part of the developing septum (corresponding to the lateral septal nucleus) (Fig. 13 C) Medially in this area, the fibres were aggregated into more dense islands (Fig 13 B)

The distribution of CA fibres in the olfactory tubercle and the septal region of the human fetus is notably similar to that of the rat brain. Thus, the bed nucleus of the stria terminalis, the nucleus accumbens, and the olfactory tubercle (including some of the islands of Calleja) are densely innervated by CA fibres also in rat, [30, 33, 68] and the distribution of CA fibres in the septum and the nucleus of the diagonal band in the human fetus resembles that described in rat [68] In rat, the olfactory tubercle the nucleus accumbens, and part of the bed nucleus of the stria terminalis are innervated by a separate portion of the ascending dopaminergic neuron system of the mesencephalon[3, 73]

C. Frontal section. Delicate varicose CA-fibres in the head of the caudate nucleus, close to the internal capsule (cf Fig. 1 L) (X 140)

D. Frontal section. Scattered CA fibres in an area of the caudate nucleus more distant from the internal capsule than in C. (X 110)

E. In the developing fetal putamen bulbous swellings t the end of blended fluorescent fibres occur possibly identical to growth cones (X 240)

F Picture showing the coarser type of CA fibre (arrow) present among the more delicate ones in the putamen. It is suggested that this fibre type might contain noradrenaline (X 260)

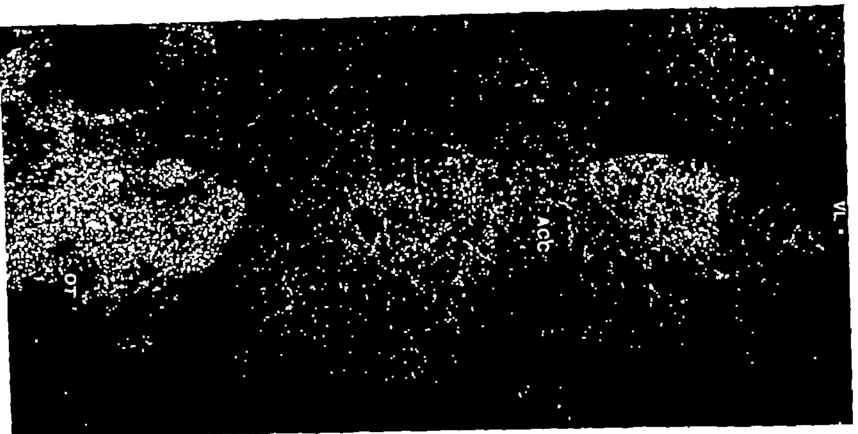


Fig. 12 Frontal section. Ph tomontage of n. accumbens (ACC) and part of the olfactory tubercle (OT) at a level close to the one shown in Fig. 1 M. The lateral ventricle is seen in the upper part of the picture (VL) (X 80)

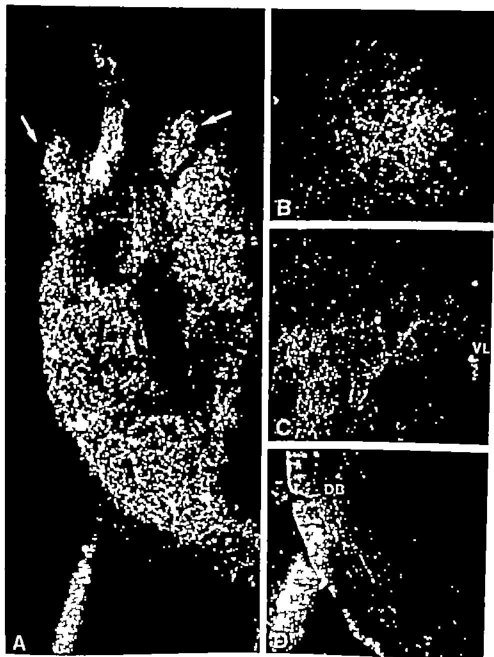


Fig 13 Frontal sections through the olfactory tubercle and septal region (cf. Fig 1 M)
A. The dense supply of delicate varicose green-fluorescent fibres in the medial part of the olfactory tubercle. The fibre aggregations in the upper part of picture are probably identical to islands of Calleja (arrow) (× 140)
B. Aggregations of fluorescent varicose fibres in the medial part of the lateral septal nucleus. (× 110)
C. CA fibres in the lateral septal nucleus close to the lateral ventricle (VL) (× 130)
D. Parallely arranged varicose fibres present within the nucleus of the diagonal band (DB). These fibres continue dorsally into the medial part of the septum. (× 130)







Fig. 14. A. Frontal section through the bed nucleus of the stria terminalis. This nucleus contains a dense innervation of delicate varicose CA fibres. Part of the anterior commissure is seen in the lower part of the picture (AC) (× 140)

B. Intensely fluorescent CA-containing fibres in the pineal gland of the human fetus. These fibres are probable noradrenaline-containing postganglionic sympathetic fibres. P = parenchyma of the pineal gland (× 140)

C. Sympathetic noradrenaline-containing nerves surrounding a pial vessel (× 160)

The similar appearance and the pparent continuity of the CA innerva tions in these areas in the neostriatum of the human fetus would be consistent with such a common site of origin also in the human l rain

B. *Occurren e of peripheral sympathetic fibr*

Intensely fluorescent, rather coarse, CA-containing fibres most probably identical with noradrenaline-containing postganglionic sympathetic fibres, were observed in association with larger vessels at the l rain surfaces (Fig 14 C) and within the pineal gland (Fig 14 B) Most of them had a smooth appearance and were confined to bundles of varying thickness running in the connective tissue surrounding the vessels and the parenchyma of the pineal From such bundles fibres l ranched into the outer portion of the vessel walls or into the parenchyma of the pineal Some of these branching fibres l ad a vari-cose appearance similar to that of mature sympathetic terminals Innervation of pial vessels and pineal gland by sympathetic adrenergic neurons has pre viously been demonstrated in several laboratory mammal 40, 41 71 73 The pinealocytes of the fetal pineal did not exhibit any monoamine fluorescence (*cf* Fig 14 B)

DISCUSSION

The present study demonstrates the localization of catecholamines and indol amines in cell bodies, axons, and axon terminals of neurons in the human brain. This has previously been extensively documented for many sub-human species (for reviews see *g* ref 31 32 56 78) In the 3—4 month human fetus, the fluorescence histochemistry of the monoamine neurons revealed an advanced stage of development f cell bodies and principal axon pathways whereas the patterning of the axon terminals is only partially developed. Thus, the most immature regions, such as the cerebral and cerebellar cortices, had no, or only very few detectable CA-contain ng fibres in contrast to, *e.g* the basal ganglia the hypothalamus nd the septal region, which are at a more advanced stage of development and exhib t well-developed patterns of probably terminal varicose CA-contain ng fibres. The very low concentrations of noradrenaline nd dopam ne (between 10 and 20 ng/g of tissue) in the whole brains of th 3—4 month old fetuses are thus primarily due to the limited developme t of the t rminal axonal networks. The overall stage of development of the monoam ne neuron sy tems n the studied fetuses seems to be comparable to that of th rat d ri g the first or second w ek after birth, a tage when the principal features of th topogr aphy of the n CA neuron systems have been establish d 80 83 84 The present material an thus provide much information about the organization of cell bodies and xon path ways of the central mono-amine neurons in the h man where the information on the terminal distributions and n ervation patter of these neurons is incomplet nd restric ted to the basal gangli the h pothalamus, and the septal and lfactory regions

Although there are many differences with respect to details, it is notable that the principles of organization of the monoamine neuron systems are quite similar in the human and the rat brain Thus, the distribution and grouping of cell bodies in the medulla oblongata and the lower brain stem and the course of the main ascending and descending axon bundles are comparable in the two species. Also the distribution of CA terminals — to the extent that they had developed in the studied human specimens — shows definite similarities to that of the rat brain.

The fluorescence microscopical demonstration of a nigrostriatal CA pathway in the human brain is of particular interest. Although there is much indirect evidense for such a system also in man, *e g* from observations in parkinsonian patients (see ref 37) its actual demonstration has been lacking Due to the strong fluorescence of the monoamine-containing fibre bundles in the fetuses, the nigro-striatal system could be mapped from the substantia nigra into the internal capsule and the globus pallidus. The course of the fibres revealed in this way has clear similarities to that described by Carpenter and Peter [22] in the monkey and by Moore *et al.* [57] in the cat. In the human fetuses, CA-containing varicose fibres were demonstrated not only in the caudate nucleus and the putamen, but also in the developing globus pallidus. This conforms well to the biochemical findings of moderate to high concentrations of dopamine in these regions in adults [5, 6, 25, 29, 37, 75] and in an 8 months old human fetus published by Bertler [5] As the globus pallidus appears to be devoid of CA-containing terminals in the rat, [30, 52] it has been questioned whether the dopamine present in this structure in man was confined to non-terminal fibres passing through the nucleus to the neostriatum or to actual terminals innervating the nucleus [37] The present finding provide evidence for a direct CA (probably mainly dopamine-containing) fibre supply to the globus pallidus suggesting that in man the mesencephalic dopamine-containing neurons might project also to globus pallidus. Nigro-pallidal fibre connexions have been presumed to exist in the human brain [37] but neither in cat [57] nor in monkey [22] a substantial termination of nigral fibres has been possible to demonstrate in the globus pallidus with silver staining of degenerating ter minals. Thus, until direct evidence for a nigro-pallidal projection has been obtained in man, the possibility has to be considered that the CA fibres in the globus pallidus originate in other areas, *e g* in mesencephalic CA cell bodies outside the substantia nigra.

The present observations might also be of significance for the interpretation of the findings of varying but significant, levels of dopamine and noradrenaline in the nucleus ruber in the human brain [5, 28, 75] As in the rat, this nucleus was devoid of CA-containing structures in the human fetus, but the ascending CA bundles from medulla pons and from mesencephalon passed close to the nucleus in a capsule-like arrangement (see Figs 1 G and 6 A) These ascen-ding CA-bundles thus seem to be the likely source of the CA:s detected in preparations of the nucleus ruber

The second major probably ascending, CA fibre system could be traced in

the human fetuses from the medulla oblongata up to the septal region, and it received fibres along its course from the cell bodies in medulla oblongata, pons (i.e. locus coeruleus and the subcoeruleus area) and mesencephalon. This pathway corresponds to an ascending fibre system in the rat, which is composed of several components, among others, major noradrenaline fibre systems originating in the cell groups of the pons and medulla oblongata and projecting to diencephalic and cortical areas [2, 15, 32, 66, 78]. In the rat, this fibre system is separated into a dorsal and a ventral bundle in the mesencephalon but no such distinct separation could be observed in the human fetus. It seems probable that this difference is due to the position of the nucleus ruber which is much larger in man, making the ascending CA fibres appear not in distinct bundles but rather as a capsule of fibres sweeping arount the nucleus (cf. Figs. 1 F and G).

Hypothalamus is one of the probable areas of termination of the ascending CA fibre system from the medulla and pons, being the region with the highest concentrations of noradrenaline in the brain of the adult [5, 8, 29, 37] as well as of the 8 months old fetus [5]. This corresponded to a rich supply of varicose CA containing fibres in several hypothalamic nuclei in the present 3—4 months old fetuses. Apart from this extrinsic, probably noradrenergic, innervation, there occur in the rat diencephalic noradrenaline and dopamine-containing cell groups [15, 32] that most probably have intrinsic diencephalic projections [15, 16]. Björklund and Nobin [18] have distinguished four such cell groups, and of these, only cells corresponding to the tubero-hypophyseal DA system (group A12) were detected in the human fetuses. In the rat, these neurons have their terminals in the median eminence, the neural lobe, and the pars intermedia [11, 16, 41, 47] and from the present fluorescence histochemical findings, it seems probable that the organization is similar in man with respect to the innervation of the median eminence and the neural lobe. But no CA innervation of the pars intermedia cells was disclosed in the human fetal pituitaries [17]. The demonstration of tubero-hypophyseal CA (probably dopamine)-containing neurons in the human brain is interesting in view of the important role these neurons play in the release of hypophysiotrophic hormones from the median eminence in the rat (for reviews, see refs. 40, 53). The morphological basis for such a function thus seems to exist also in man.

ACKNOWLEDGEMENTS

This study was supported by grants from Svenska Livförsäkringsbolags Nämnd för Medicinsk Forskning, Harald and Greta Jeanssons Stiftelse, the Ford Foundation and from the National Institutes of Health, U.S. Public Health Service. (Grant No. NS 06701-07)

The skilful technical assistance of Annika Borgelin, Britt Nilsson and Eva Svensson is gratefully acknowledged.

REFERENCES

1 AMIN A. H. T B. B. CRAWFORD and J H. GADDUM The distribution of substance P and 5-hydroxytryptamine in the central nervous system of the dog *J Physiol. (Lo d)* 1954 *126* 596—618.

2. ANDÉN N E., A. DAHLSTRÖM K. FUXE, K. LARSSON L. OLSON and U UNGERSTEDT Ascending monoamine neurons to the telencephalon and diencephalon. *Acta physiol. scand* 1966 *67* 313—326.

3 BATTISTA, A., K. FUXE, M. GOLDSTEIN and M. OGAWA, Mapping of central monoamine neurons in the monkey *Experientia*. 1972 *28* 688—690

4 BÉDARD P L. LAROCHELLE, A. PARENT and L. J POIRIER, The nigrostriatal pathway: A correlative study based on neuroanatomical and neurochemical criteria in the cat and the monkey *Exp Neu ol.* 1969 *25* 365—377

5 BERTLER, A., Occurrence and localization of catechol amines in the human brain. *A ta physiol. end* 1961 *51* 97—107

6. BERTLER, A. A. CARLSSON E. ROSENGREN and B. WALDECK, A method for the fluorimetric determinations of adrenaline, noradrenaline and dopamine in tissues. *K gl. Fysiogr Sällsk Lund Förh.* 1958. *28* 121—123

7 BERTLER A. and E. ROSENGREN Occurrence and distribution of dopamine in brain and other tissues. *Experientia*. 1959 *15* 10—11

8 BERTLER, A. and E. ROSENGREN Occurrence and distribution of catechol amines in brain. *Acta physiol. scand* 1959 *47* 350—361

9 BJÖRKLUND A., A. ENEMAR and B. FALCK, Monoamines in the hypothalamo-hypophyseal system of the mouse with special reference to the ontogenetic aspects. *Z Zellforsch.* 1968. *89* 590—607

10 BJÖRKLUND A. and B. FALCK, An improvement of the histochemical fluorescence method for monoamines. Observations on varying extractability of fluorophores in different nerve fibers. *J Histochem Cytochem.* 1968. *16* 717—720.

11 BJÖRKLUND A. B. FALCK, F HROMEK, CH. OWMAN and K. A. WEST Identification and terminal distribution of the tubero-hypophyseal monoamine fibre systems in the rat by means of stereotaxic and microspectrofluorometric techniques. *Brain Res.* 1970. *17* 1—23

12. BJÖRKLUND A., B. FALCK and CH. OWMAN Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic amines. In, *Methods of Investigative nd Diagnostic Endocrinology* Ed. S. A. Berson. Vol. 1 *The Thyroid and Biogenic Amines.* Eds. J E. Rall and I J Kopin. North-Holland Publ. Comp., Amsterdam, 1972 p 318—368.

13 BJÖRKLUND A., B FALCK and E. ROSENGREN Monoamines in the pituitary gland of the pig *Lif S i.* 1967 *6* 2103—2110

14 BJÖRKLUND A., B. FALCK and U STENEVI Classification of monoamine neurones in the rat mesencephalon Distribution of a new monoamine neurone system. *Brain Re* 1971 *32* 1—17

15 BJÖRKLUND A., O LINDVALL, A. NOBIN and U STENEVI The adrenergic innervation of the thalamus as revealed by the glyoxylic acid fluorescence method. *Comm. Dept Anat (L d)* 1973 *4* 1—30

16. BJÖRKLUND A., R. Y MOORE, A. NOBIN and U STENEVI The organization of tubero-hypophyseal and reticulo-infundibular catecholamine neuron systems in the rat brain. *Brain Res.* 1973 *51* 171—191

17 BJÖRKLUND A. and A. NOBIN unpublished observations.

18. BJÖRKLUND A. and A. NOBIN Fluorescence histochemical and microspectrofluorometric mapping of dopamine and noradrenaline cell groups in the rat diencephalon. *Brain Res.* 1973. *51* 193—205.

19 BJÖRKLUND A., A. NOBIN and U STENEVI Effects of 5,6-dihydroxytryptamine on nerv terminal serotonin and serotonin uptake in the rat brain. *Brain Res.* 1973. *53* 117—127

20. BJÖRKLUND A., A. NOBIN and U STENEVI The use of neurotoxic dihydroxytryptamines as tools for morphological studies on central indolamine neurons. *Comm Dept. Anat (Lund)* 1973. *3* 1—20.

21 BOGDANSKI, D F H. WEISSBACH and S. UDENFRIEND The distribution of serotonin, 5-hydroxytryptophan decarboxylase, and monoamine oxidase in brain. *J Neurochem* 1957 *1* 272—278.

22 CARLSSON A., B. FALCK and N.-A. HILLARP Cellular localization of brain monoamines. *Act physiol. scand* 1962, *56*, Suppl. 196. 1—28.

23. CARPENTER, M. B. and P PETER, Nigrostriatal and nigrothalamic fibres in the rhesus monkey *J Comp. N urol.* 1972. *144* 93—116.

24 DAHLSTOM A. and K. FUXE, Evidence for the existence of monoamine-containing neurons in the central nervous system. I: Demonstration of monoamines in the cell bodies of brain stem neurones. *Acta physiol. scand* 1964 *62* Suppl. 232. 1—55.

25. DAHLSTRÖM A. and K. FUXE, Evidence for the existence of monoamine neurons in the central nervous system. II: Experimentally induced changes in the intraneuronal amine levels of bulbospinal neuron systems. *Acta physiol. scand* 1965. *64* Suppl. 247 1—36.

26. DALY J K. FUXE and G. JONSSON Effects of intracerebral injections of 5 6-dihydroxytryptamine on central monoamine neurons Evidence for selective degeneration of central 5-hydroxytryptamine neurons. *B ain Res.* 1973 *49* 476—482.

27 DE LA TORRE, J C., Catecholamines in the human diencephalon: A histochemical fluorescence study *Act neur path. (Berl.)* 1972. *21* 165—168

28. EHRINGER, H. and O. HORNYKIEWICZ, Verteilung von Noradrenalin und Dopamin (3-Hydroxytyramin) im Gehirn des Menschen und ihr Verhalten bei Erkrankungen des extrapyramidalen Systems. *Wien klin. W chr* 1960. *38* 1236—1239

29 FAHN S., L. R. LIBSCH and R. W CUTLER, Monoamines in the human neostriatum topographic distribution in normals and Parkinson's disease and their role in akinesia, rigidity chorea and tremor *J neurol. Sci.* 1971 *14* 427—455.

30. FUXE, K. Evidence for the existence of monoamine neurons in the central nervous system. IV The distribution of monoamine terminals in the central nervous system. *A t physiol. scand* 1965 *64* Suppl. 247 37—85.

31 FUXE, K., T HÖKFELT and U UNGERSTEDT Localization of indolealkylamines in CNS. *Advanc Pharmacol.* 1968. *6* 235—251

32. FUXE, K., T HÖKFELT and U UNGERSTEDT Distribution of monoamines in the mammalian central nervous system by histochemical studies. In, *Metabolism of Am nes the B ain.* Ed. E. Hooper Mac Millan London, 1969 p. 10—22.
33 FUXE, K. and CH. OWMAN Cellular localization of monoamines in the area postrema of certain mammals. *J Comp Ne rol.* 1965. *125* 337—354
34 GOLDEN G S. Embryologic demonstration of a nigro-striatal projection in the mouse. *Brain Res* 1972. *44* 278—282.
35 HELLER, A., J A. HARVEY and R. Y MOORE A demonstration of a fall in brain serotonin following central nervous system lesions in the rat. *Biochem Pharmacol* 1962. *11* 859—866.
36. HOCHSTETTER, F Beiträge zur Entwicklungsgeschichte des menschlichen Gehirns. Franz Deuticke Wien und Leipzig, 1929
37 HORNYKIEWICZ, O Dopamine (3-hydroxytyramine) and brain function. *Pharm col. R* 1966 *18* 925– 964
38. HYYPPÄ M., Differentiation of the hypothalamic nuclei during ontogenetic development in the rat. *Z. Anat Entwickl.-Gesch.* 1969 *129* 41—52.
39 HAGGENDAL, J An improved method for fluorometric determination of small amounts of adrenaline and noradrenaline in plasma and tissues. *A ta physiol scand* 1963 *59* 242—254
40 HÖKFELT T and K. FUXE On the morphology and the neuroendocrine role of the hypothalamic catecholamine neurons. In *B ain-Endocrine Interaction. M dian Eminen e Structure and Functi n.* Eds. K. M. Knigge, D E. Scott and A. Weindl. Karger Basel 197 p 181—223
41 JONSSON G K. FUXE and T HÖKFELT On the catecholamine innervation of the hypothalamus with special reference to the median eminence. *B ai Res.* 1972. *40* 271—281
42. JOYCE D Changes in the 5-hydroxytryptamine content of rat, rabbit and human brain after death. *Brit. J Pharmacol.* 1962. *18* 370—380
43 KUHAR, M J G K. AGHAJANIAN and R. H. ROTH, Tryptophan hydroxylase activity and synaptosomal uptake of serotonin in discrete brain regions after midbrain raphe lesions Correlations with serotonin levels and histochemical fl orescence. *Brain Res.* 197? *44* 165—182.
44 KUHLENBECK, H., The human diencephalon. *Confinia Neurologica* 1954 *14* (S ppl.)
45 LEFRANC G A. L L HERMITE and J TUSQUES, Mise en évidence de neurones monoaminergiques par la technique de fluorescence dans l'encéphale d'Anguille. *C R. Soc Biol.* 1969 *5* 1193—1196
46. LÉONARDELLI J and E. HERMAND Étude des fibres et des neurones monoaminergique de l'hypothalamus chez le Hamster doré. *C R. Soc Biol.* 1967 *161* 184—2188.
47 LICHTENSTEIGER, W and H. LANGEMANN Uptake of exogenous catecholamines by monoamine-containing neurons of the central nervous system Uptake of catecholamines by arcuato-infundibular neurons. *J Pharmacol. exp. Ther* 1966 *151* 400—408.
48. LINDVALL, O and A. BJÖRKLUND unpublished observations.
49 LOIZOU L. A. The postnatal development of monoamine-containing structures in the hypothalamo-hypophyseal system of the albino rat. *Z Zellforsch.* 1971 *114* 234—252.

50. LOIZOU L A., The postnatal ontogeny of monoamine-containing neurones in the central nervous system of the albino rat. *Brain Res.* 1972. 40 395—418.

51 MAEDA, T and A. DRESSE, Recherches sur le développement du locus coeruleus. *Act neurol belg* 1969 69 5—10

52. MAEDA, T and N SHIMIZU Projections ascendantes du locus coeruleus et d'autres neurones aminergiques pontiques au niveau du prosencephale du rat *Brain Res.* 1972. 36 19—36.

53. McCANN S. M., P S. KALRA, A. O DONOSO W BISHOP H P G SCHNEIDER C. P FAWCETT and L KRULICH The role of monoamines in the control of gonadotropin and prolactin secretion. In, *Brain-Endocri Interaction. M dia Emi ence Structure and Function* Eds. K. M Knigge, D E. Scott and A. Weindl. Karger Basel 1972 p 224—235.

54 McGEER, E. G and P L. McGEER, Catecholamine content of spinal cord *Canad. J Biochem. Physiol* 1962. 40 1141—1151

55. METTLER, F A., Anatomy of the basal ganglia. In, *Handbook of Cl ical N urology* Vol. 6: *Diseases of the Basal Ganglia* Eds. P J Vinken and G W Bruyn. North Holland Publ. Comp. Amsterdam, 1968, p. 1—55.

56. MOORE, R. Y., Brain lesions and amine metabolism. *Int Rev Neurobiol* 1970. 13 67—91

57 MOORE, R. Y R. K. BHATNAGAR and A. HELLER, Anatomical and chemical studies of a nigro-neostriatal projection in the cat. *Brai Res.* 1971 30 119—135.

58. MOORE, R. Y., A. BJORKLUND and U STENEVI Plastic changes in the adrenergic innervation of the rat septal area in response t denervation. *Brai Res.* 1971 33 13—33

59 NAUTA, W J H and W HAYMAKER, Hypothalamic nuclei and fiber connections. In, *The Hypothalamus.* Eds. W Haymaker E. Anderson and W J H. Nauta. Charles C. Thoma Springfield, Illinois, 1969 p. 136—?09

60. NIELSEN K. C. and CH. OWMAN Adrenergic innervation of pial arteries related to the circle of Willis in the cat. *Brain Res* 1967 6 773—776.

61 NIELSEN K. C., CH OWMAN and B. SPORRONG Ultrastructure of the autonomic innervation apparatus in the main pial arteries of rats and cats. *Brain Res* 1971 27 25—32.

62. NOBIN A., H.-G. BAUMGARTEN A. BJORKLUND I LACHENMAYER and U STENEVI Axonal degeneration and regeneration in the bulbospinal indolamine neuron systems after 5 6-dihydroxytryptamine treatment. *Brain Res.* 1973 56 1—24

63 NYSTROM B., L. OLSON and U UNGERSTEDT Noradrenaline nerve terminals in human cerebral cortices. First histochemical evidence. *Scien* 1972. 176 924—926.

64 ODAKE, G Fluorescence microscopy of the catecholamine containing neurons of the hypothalamo-hypophysial system. *Z. Zellforsch.* 1967 8. 46—64

65 OLSON L. and K. FUXE, On the projections from the locus coeruleus norad renaline neurons The cerebellar innervation. *Brain Res.* 1971 28 165—171

66 OLSON L. and K. FUXE, Further mapping out of central noradrenaline neuron systems Projections of the subcoeruleus area. *Brain Res.* 1972. 43 289—295.

67 OLSON L., A. SEIGER and K. FUXE, Heterogeneity of striatal and limbic dopamine innervation Highly fluorescent islands in developing and adult rats. *Brai Res.* 1972. 44 283—290

68. OLSON L. and Å. SEIGER Early prenatal ontogeny of central monoamine neurons in the rat: Fluorescence histochemical observations. *Z. Anat Entwickl. Gesch.* 1972. *137* 301—316.
69 OLSON L. and U UNGERSTEDT A simple high capacity freeze-drier for histochemical use. Histochemie 1970 22 8—19
70 OLSZEWSKI J and D BAXTER, *Cytoarchitecture of the Human Brain Stem.* Karger Basel, 1954
71 OWMAN CH., Localization of neuronal and parenchymal monoamines under normal and experimental conditions in the mammalian pineal gland. In, *Progress in Brain Research.* Vol. 10 *Structur nd Functio of the Epiphysis Cerebri.* Eds. J A. Kappers and J P Schadé. Elsevier Publ. Comp., Amsterdam—London—New York, 1965 p 423—453
72. OWMAN CH., Sympathetic nerves probably storing two types of monoamines in the rat pineal gland. *I t J Neuropharmacol.* 1964 2 105—112.
73 RILEY H. A. R., *An Atlas of the Basal Ganglia Brai Stem and Spinal Cord* The Williams and Wilkins Company Baltimore, 1943
74 RUSSEL, G. V The nucleus locus coeruleus (dorsolateralis tegmenti) *Texas Rep. Biol. Med* 1955 *13* 939—988.
75 SANO I T GAMO Y KAKIMOTO K. TANIGUCHI M. TAKESADA and K. NISHINUMA Distribution of catechol compounds in human brain. *Biochim. Biophys. Acta (Amst)* 1959 *32* 586—587
76 SMITH, G G. and R. W SIMPSON Monoamine fluorescence in the median eminence of foetal neonatal and adult rats. *Z Zellforsch.* 1970 *104* 541—556.
77 TENNYSSON V M., R. E. BARRETT G COHEN L. COTE, R. HEIKKILA and C. MYTILINEOU The developing neostriatum of the rabbit Correlation of fluorescence histochemistry electron microscopy endogenous dopamine levels, and (^{3}H) dopamine uptake. *Brai Res* 1972 *46* 251—285
78. UNGERSTEDT U Stereotaxic mapping of the monoamine pathways in the rat brain. *Act physiol. scand* 1971 Suppl. 367 1—48.
79 VOGT M. The concentration of sympathin in different parts of the central nervous system under normal conditions and after the administration of drugs. *J Physiol. (Lond.)* 1954 *123* 451—481

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 389

From the Department of Medical Pharmacology
University of Uppsala Sweden

SEXUAL MOTIVATION IN THE FEMALE RAT

A METHODOLOGICAL STUDY APPLIED TO THE INVESTIGATION OF THE EFFECT OF ESTRADIOL BENZOATE

By

BENGT J MEYERSON
LEIF H LINDSTRÖM

UPPSALA 1973

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 389

*From the Department of Medical Pharmacology
University of Uppsala Sweden*

SEXUAL MOTIVATION IN THE FEMALE RAT

A METHODOLOGICAL STUDY APPLIED TO THE INVESTIGATION OF THE EFFECT OF ESTRADIOL BENZOATE

By

BENGT J MEYERSON
LEIF H LINDSTRÖM

UPPSALA 1973

CONTENTS

CHAPTER I

THE NATURE AND SCOPE OF THE PROBLEM

Most investigations of the influence of hormones on female sexual behavior have been focused on copulatory behavior. It is established that copulatory behavior in female lower mammals is dependent on ovarian hormones. In some species, the behavior is restored by estrogen administration after ovariectomy whereas, for instance, in the rat, hamster and guinea-pig, progesterone has to be given as well (see Young, 1961). It is likely that the performance immediately related to the copulatory act is preceded by a specific desire (drive) in the female animal to seek sexual contact. The concept of sexual motivation will, in the following, be used in this sense: a physiological state which brings the animal to actively seek sexual contact with another animal. The significance of gonadal hormones in the production of sexual motivation has been studied less than the effects of hormones on copulatory behavior. This fact might partly be due to methodological difficulties. Copulatory behavior comprises easily definable motor patterns which the animals do not display except in connection with the copulatory act. However, the intensity of the copulatory act or the readiness to respond to mating attempts of another individual cannot be taken as a measure of sexual motivation. It is the eagerness to seek sexual contact and not the consummatory act which interests us in the following.

In the early studies by Moss (1924), Warner (1927), Jenkins (1928) and Nissen (1929) a possible relationship was indicated between ovarian hormones and the tendency of a female rat to approach a male rat, measured in terms of the number of times the subject crossed an electrically charged grid to reach the male (Columbia obstruction method). Direct contact between the tested female and the incentive animal was allowed each time the subject crossed the electric grid. After crossing the grid the female was brought to the starting box and the experiment started again and lasted for a predetermined time period of 20 min. Warner demonstrated that females with vaginal smears in the cornified state crossed the electric grid on the average 10 times as often as in diestrus. In females with cornified smears a male was shown to be more of an incentive than an empty cage. Nissen showed, using the same apparatus, that the females' willingness to cross the electric grid to reach a male declined after ovariectomy but could be restored by extract from cow placenta. The effects of sex segregation upon the sexual drive measured by the electrical obstruction technique was investigated by Jenkins (1928). He found that segregation at the age of 30 days decreased the number of grid crossings in intact estrous

(cornified vaginal smears) rats when the incentive was a male In contrast the number of grid crossings increased compared to non-segregated females when the incentive was a diestrous female

More recently the time to run down a runway was used as a measure of the urge to seek sexual contact (Bolles *et al.* 1968) It was found that estrous females run faster than diestrous controls but running times were not better when the female ran towards an active than towards a sexually passive male It should be noted that the "passive" male was not castrated. Data are very scant as to the reinforcing value of different components of sexual contact with a male such as mounting, intromission ejaculation etc to the female rat In estrous females trained to press a lever for sexual contact (copulation) it was demonstrated that intervals between responses (lever pressings) increased with increasing sexual stimulation (e.g. ejaculation-response latencies were longer than mount response latencies, (Bermont 1961 Bermont and Westbrook 1966). Escape following sexual contact was demonstrated by Peirce and Nuttall (1961) and Rogers (1967), indicating that females might also work to avoid a sexual stimulus. It seems that the interval between sexual contacts is important and this was also suggested by Bermont. During the latency period which is dependent on the intensity of the sexual stimulus, sexual contact might be aversive Aversive components of coital stimulation was also recently demonstrated by Hardy and DeBold (1972)

In the light of earlier studies the significance of ovarian hormones in the production of sexual motivation in the female rat seems plausible Today's access to pure hormone preparations, hormone-analogues of different structural configurations anti-hormones, and modern neuropharmacological compounds means that new tools are available for further investigations of the neuroendocrinology of the sexual drive It seems desirable that measurements of as complex a matter as sexual motivation should involve more than one technique It was therefore one of the aims of the present investigation to develop several techniques and routines suitable for measuring sexual motivation

SYNOPSIS OF THE TECHNIQUES USED

Three different methods were employed the open field increasing barrier and runway choice methods.

In the *OPEN FIELD* method the animals were observed in an observation arena The incentive animals were placed in mesh-cages which were located in the wall of the arena. The subject being studied was free to move around and the location of the subject was recorded photographically at predetermined time intervals. The method records when and to what extent the subject is willing to seek contact with the incentive animal This technique has the advantage of not introducing experimental tools such as punishment or work (crossing of electric grids bar-pressing etc)

An electric grid was used as an obstruction in the *INCREASING BARRIER* method

in order to determine how much of an aversive stimulus the subject is willing to overcome to reach sexual contact. Like the Columbia obstruction method, the subject had to pass from one cage via the electric grid to a second cage which held the incentive animal. However, in contrast to the Columbia technique the grid current was not kept constant but increased stepwise every second time the animal crossed. The experiment lasted until the animal was no longer willing to cross.

The third method, the *RUNWAY CHOICE* was designed to give information about the specific choice between two different incentive objects made by the tested animal. The animal was placed in a runway which led the animal to a choice situation in a chamber with two doors at the far end. Each door led into a separate goal-cage. In the goal-cage the subject met the specific incentive animal.

A detailed description of the procedures and apparatus used is given in the following.

GENERAL METHODS

A CATEGORIES OF ANIMALS

Experimental subjects

Female Sprague Dawley rats all from the same supplier (Anticimex, Sollentuna, Sweden) were used throughout. They were weaned and the two sexes separated at 22 days of age. Batches of 60–100 animals (250 g, 4 months of age) were purchased at a time. They were used for experimentation for 6 months. All the methods were run in parallel: animals from the batch were randomly assigned to each method and were used only in that method. If not otherwise stated the animals were ovariectomized as adults and used in the experiments no earlier than three weeks after surgery.

Before being used in experiments on sexual motivation, each batch of females was tested for mating behavior following a standard treatment, estradiol benzoate (10 μg/kg s.c.) followed 48 hours later by progesterone (0.4 mg/rat s.c.). This procedure was undertaken to give the subjects mating experience and also to establish the responsiveness to estrogen treatment. The tests for mating behavior were performed essentially as previously described (Meyerson, 1964). Each animal was tested 4, 6 and 8 hours after the progesterone treatment and was subjected at each test to 6–8 mounts by a sexually vigorous male. The percentage responding was based on animals which displayed clearcut lordosis response in at least two of the three tests performed. No batch of animals used in the present investigation had less than a 70 % response.

The Incentive Animals

A pool of sexually active and inactive animals was kept in the laboratory The specific animal to be used as an incentive animal was randomly taken from this pool at each run.

Sexually Active Males. Wistar males (purchased as specific pathogen free Möllegaard, Ejby Denmark) selected for good sexual activity were used Their sexual vigour was maintained by at least one weekly opportunity to copulate with females in heat

Sexually Active Females. Spayed Sprague-Dawley females were kept in heat by estradiol benzoate (25 μg/rat) followed 48 hours later by progesterone (1 mg/rat). The females were used as incentive subjects 6—10 hours after the progesterone injection

Sexually Inactive Males and Females. The same kind of animals were used as above Males had been castrated for at least 2 months and females at least 1 month before being used as incentives.

B. CARE OF ANIMALS AND ENVIRONMENTAL CONDITIONS

The animals were kept two or three in the same cage (Macrolon[R] 42 x 27 x h 15 cm) and they were housed and tested in windowless laboratories artificially illuminated with 12 hours dimmed light and 12 hours bright light The temperature was maintained between 22–24 C. Standard laboratory chow (No 210 Anticimex) and water were freely available Females and males were kept in the same room but segregated to the extent that care was taken not to keep the male and female cages in the immediate vicinity of each other To dampen changes in exogenous noise during working hours a broadcasting program was supplied day and night to all rooms. The noise level was just sufficient for the human ear to catch a broadcast conversation

Testing Room The experiments were performed in rooms separate from those in which the animals were housed. Each method was carried out in a different room. Nobody but the observer who was running the experiment was in the testing room during the experiment The rooms were windowless and the only light source was a dimmed white light sufficient to permit the observer to make his records.

C. THE STIMULUS SITUATION

Throughout the present investigation, if not otherwise stated, sexual contact was restrict ed i.e. contact with the stimulus animal was allowed but the animals were separated by a wire mesh.

The incentive animals actively sought contact regardless of category when the experimental subject approached the separating mesh. Sluggish incentive animals were immediately replaced Different stimuli were used viz. a vigorous male a castrated male an estrous female or an empty cage These measures were taken to enable a conclusion to be made as to whether an effect was predominantly sexual or if it was due to increased locomotor activity social behavior or some other factor

The empty cage" was identical to the one used in the apparatus to house the incentive animal but had never been used for animals on any occasion. In addition the cage holding the incentive animals was restricted to only one category of stimulus animal This was done to avoid a possible influence of persistent odor

D THE VAGINAL SMEAR RECORDS

Vaginal smears were taken by the lavage technique (Zarrow *et al.* 1964) and examined immediately without staining. The smears were scored in the following way according to the dominating cell type D (diestrous) = leucocytes, P (proestrous) = nucleated epithelial cells E (estrous) = cornified epithelial cells

E. INJECTED MATERIALS

17β-estradiol 3-benzoate (EB) and progesterone were dissolved in olive oil and injected in a volume of 0.25–0.35 ml. Olive oil was injected as the oil blank solution. All injections were given subcutaneously The time of injection was always 30–60 min before the start of the dark period.

F EXPERIMENTAL DESIGN

The subjects were randomly divided into groups of 4–6 members. Two groups were always run in parallel one group as a control and the other one as experimental. One *test-session* per day was conducted for a certain number of days, the *test-period*. The treatment was then repeated with the control group as experimental group and vice versa. A new test-period was not started until at least 14 days had elapsed from the last treatment. The complete experiment for each treatment category consisted of several replicate blocks of cross-over tests. Exceptions from this design will be mentioned under procedure for the specific experiment

The Incentive Animals

A pool of sexually active and inactive animals was kept in the laboratory The specific animal to be used as an incentive animal was randomly taken from this pool at each run

Sexually Active Males Wistar males (purchased as specific pathogen free Mollegaard Ejby Denmark) selected for good sexual activity were used Their sexual vigour was maintained by at least one weekly opportunity to copulate with females in heat

Sexually Active Females. Spayed Sprague Dawley females were kept in heat by estradiol benzoate (25 μg/rat) followed 48 hours later by progesterone (1 mg/rat) The females were used as incentive subjects 6–10 hours after the progesterone injection

Sexually Inactive Males and Females The same kind of animals were used as above Males had been castrated for at least 2 months and females at least 1 month before being used as incentives.

B CARE OF ANIMALS AND ENVIRONMENTAL CONDITIONS

The animals were kept two or three in the same cage (Macrolon[R] 42 x 27 x h 15 cm) and they were housed and tested in windowless laboratories, artificially illuminated with 1 hours dimmed light and 12 hours bright light. The temperature was maintained between 22–24 C Standard laboratory chow (No 210 Anticimex) and water were freely available Females and males were kept in the same room but segregated to the extent that care was taken not to keep the male and female cages in the immediate vicinity of each other To dampen changes in exogenous noise during working hours a broadcasting program was supplied day and night to all rooms. The noise level was just sufficient for the human ear to catch a broadcast conversation

Testing Room The experiments were performed in rooms separate from those in which the animals were housed. Each method was carried out in a different room. Nobody but the observer who was running the experiment was in the testing room during the experiment The rooms were windowless and the only light source was a dimmed white light sufficient to permit the observer to make his records.

C. THE STIMULUS SITUATION

Throughout the present investigation, if not otherwise stated sexual contact was restricted i.e. contact with the stimulus animal was allowed but the animals were separated by a wire mesh.

C.

Difference between two experiment (EXP) experimental block or groups within the same treatment category (d exp 1s or d con rs)

Contr 1 ⟷ Contr 2

Exptl 1 ⟷ Exptl 2

Comparisons made between one or the over go for several test sessions

EXP 2: exptl 2; d exptl; contr 2

EXP 1: exptl 1; d contr; contr 1

RESPONSE

TEST-SESSION No

Mann-Whitney U test

(MWU)

The scores from the two series are ranked in order of increasing size. For the procedure to calculate the U see Siegel (1956 p 116)

the scores obtained for series 1 are the equal to those of series 2

Score	20	23	35	40	72	81
from EXP	1	1	2	1	2	2

D

Difference between the effects of two treatments

d_1 (Exp 1 $_1$-Contr$_1$) ⟷ d_2

(Exp 1 $_2$-Contr$_2$)

EXP 2: exptl 2; d_2; contr 2

EXP 1: exptl 1; d_1; contr 1

TEST SESSION No

Mann-Whitney U

(MWU)

The d-values for the two series are ranked

H the d-values of the two series are equal

d-values	4	16	20	33	0	42
from EXP	1	1	1	2	2	1

1) For detailed procedure see Siegel 1956

TABLE 1 . . . analysis

G STATISTICAL ANALYSIS

The primary data for the techniques used showed a skewed distribution This skewness was in several instances found to vary between the control and the experimental data and also between the experimental data over consecutive test-sessions. This led us to choose non-parametric statistical tests.

The basic statistical treatment of the data to establish if a certain null-hypothesis should be rejected or not was carried out generally as described in Table 1 If other tests were used this will be stated in the text. The four different procedures were called A, B, C and D (see Table 1) and the different tests were Fr = Friedman two-way analysis of variance W = Wilcoxon matched pairs signed-ranks test and MWU = Mann Whitney U-test (see Siegel 1956) The kind of statistical analysis used in the text and tables will be referred to in the following way Procedure A Friedman s test = A.Fr $p < 0.02$ etc. Not significantly different (NS) means $p > 0.05$

CHAPTER II

STUDIES ON SEXUAL MOTIVATION IN THE FEMALE RAT BY MEANS OF THE OPEN FIELD TECHNIQUE

The essential principle of the open field technique is to study the behavior of the animal when it is free to move in an open area which also holds the incentive animal This method measures mainly the amount of time the subject spends within a certain distance from the stimulus animal. We assume that this time is related to the subject s sexual or social motivation to seek contact with the incentive animal

The advantage of the open field technique in studying goal directed behavior is that the observations involve comparatively few disturbances by such factors as the physical structure of the apparatus or acquisition of a certain behavior performance pattern necessary to reach the goal. The open field procedure has in most cases implied that the subject is introduced into a novel environment (Broadhurst 1958 Candland and Campbell, 1962) In contrast in the present open field procedure care was taken to minimize responses to irrelevant stimuli in the environment by adapting the subjects to the apparatus for a certain time before the experiment started

A survey of the experiments conducted is given in Table

METHODS

THE APPARATUS

A general plan of the apparatus is shown in Fig. 1 The animals were observed in a fairly sound proof box (155 x 155 x 230h cm), (double walls the space between the walls filled with rock-wool) on a circular observation arena (diameter 130 cm) The incentive animal was placed in a mesh cage in the center of the field (stimulus cage Y Fig. 1). A wall ran straight across the field dividing it into two observation areas, where two animals could be tested simultaneously In the circumference of each semicircular field was located a mesh cage which held another incentive animal (stimulus cage X, Fig 1). An automatic 16 mm movie camera, equipped with a wide-angle objective was placed in the cage ceiling. The camera was triggered at intervals by a preset timing device. A counter triggered at the same time as the camera was photographed in order to show the number of the records taken. In order to observe the animals in dimmed light an

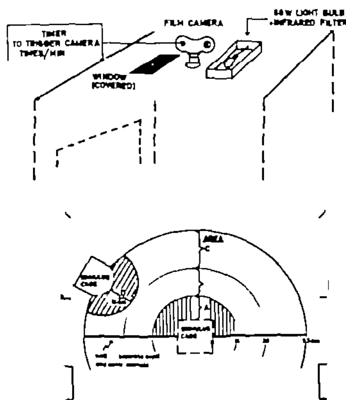


Figure 1 The OPEN FIELD APPARATUS For description f details see text under apparatus.

OPEN FIELD FLOW-SCHEDULE OF PROCEDURE

1 Subjects were placed in the field two animals were run simultaneously placed randomly in one of the two halves of the field.

Camera triggered once very minute for 60 min. = *TEST-SESSION*

3 Film record read location and activity scored

infrared sensitive film (Kodak, Infrared sensitive 248) was used If not otherwise stated the only light source in the cage was a 60 W light bulb behind an infrared filter (Wratten No 89) The light bulb was placed outside the roof of the box and the filter covered a 10 x 10 cm hole through which the light entered the box. The amount of visible light passing into the box was just enough to see the animal with the naked eye

RECORDING OF LOCATION

The field was covered by a lucite ceiling on which was painted thin lines to show the borders of four areas A, B C and D (see Fig 1) The projection of these lines on the floor of the field had the distances from the cages X and Y as described in Fig. 1 The film was read in a viewer editor apparatus. The location of the animal was scored according to the area in which the head of the rat was located Location is expressed as the percentage of records in which a subject was located in a certain area Data which appear in figures and tables are mean values (see also flow-schedule of the procedure)

THE RECORDING OF LOCOMOTOR ACTIVITY

A rough estimate of locomotor-activity was obtained by the percentage of records in which an animal was found to have changed position between two consecutive film records. New position means that the head had moved 5 cm from its previous position regardless of direction. Data on activity which appear in the figures and tables are mean values.

TRAINING AND ADAPTATION TO THE APPARATUS

The subjects were adapted to the experimental situation by being left in the field for a couple of hours a day for about 2–3 days before the experiment started No incentive animal was placed in the apparatus during the adaptation period

FREQUENCY OF OBSERVATIONS AND TIME OF DAY RUNS WERE CONDUCTED

If not otherwise stated the camera was triggered once every minute Test-session lasted for 60 min and were conducted during the dark period of light regime (light from 9 p.m to 9 a.m) 3–8 hours after the light had been switched off

	EXP No	INCENTIVE ANIMAL	TREATMENT	NUMBER OF ANIMAL TESTED
				
Performance f the subj in the fi ld	1	Y ♂	none	8
		X ♀		
Loca ion and activity				
diff rent times within one te t- ses ion	2 b	Y ♂	none	11
		X ♀		
b after daily t- session				6
The ffect f				
ligh	3,a b	Y none	none	8
b noise		X none		12
time f the dark period ests were performed	3	Y ♂	none	4+4
		X ♀		
The trous cycl	4	Y ♂	none	7
		X ♀		
The ff ct f EB	5	Y ♂	EB 25 µg/kg	18
		X ♀	oil blank	
	6	Y ♀	EB 25 µg/kg	14
		X ♂		
	7	Y ♂	EB 25 g/kg	6
		X none		
	7 b	Y ♀	B 25 µg/kg	6
		X none		
	8	Y ♂	EB, 25 10,2 5 µg/kg or il blank	10
		X ♀		
	9 10	Y ♂	EB 10 µg/kg (9)	20
		X ♀	EB 2 5 µg/kg (10)	12

♂ sexuall ac ive mal ♀ sexually inactive female

TABLE 2. Survey f xperimen on the behavior f ovariectomized at i the OPEN FIELD apparatus

STUDIES ON THE BEHAVIOR OF THE SUBJECT IN THE OPEN FIELD

EXP 1 and 2

The possibility of measuring sexual motivation by the open field technique would be very limited if the animals were uneasy in the field e.g. just sitting in a certain area because they felt too anxious to move. The purpose of experiments 1 and 2 was to investigate the general behavior of the animal when placed in the field (*EXP 1*) and the effect of the experimental procedure on location and activity (*EXP 2*)

PROCEDURE

Untreated spayed females which had previously been adapted to the field were observed for one hour. The number of animals used in each experiment is shown in Table 2. Records were taken every minute by direct observation (EXP 1) via a small window (10x15 cm) located in the cage ceiling close to the camera or by means of the movie camera (EXP 2). The infrared filter (see Apparatus) through which the white light filtered before it was let into the cage was displaced (EXP 1) in such a way that sufficient visible light leaked into the cage to make it possible for the observer to record the behavior of the animal The cage in the center area (Y) held an intact male and the cage (X) in area D a spayed female

RESULTS

EXP 1 Performance of the subject in the field

When the subject was placed in the field it immediately started to run about more or less continuously exploring details of the field sniffing at the floor rearing against the walls etc. This initial high general exploring activity (Fig. 2) lasted for about 5 minutes After that time the animal became more oriented towards the cages of the incentive animals running from one cage to the other After about 10—15 minutes there was an obvious decline in the general exploring activity and more time was spent slowly walking around in the field with longer time periods spent in each place. When in the area of an incentive animal the subject reared against the stimulus cage or stood close to the wire mesh mainly sniffing at the incentive animal. In the later part of the observation period (after about 30 min.) the subject was sometimes seen to be just lying down. These brief periods of sitting or lying still, doing nothing or grooming, licking its fur etc were suddenly interrupted by the subject moving to another place Throughout the one-hour observa

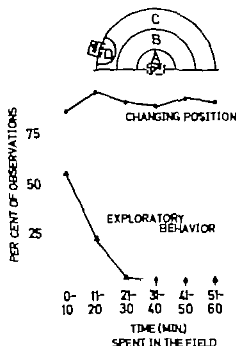


Figure 2 EXP 1 Activity of the subject in the open field recorded by direct observation. The observations were done once per min for 60 min. N 8 Changing position the subject has moved (> 5 cm) from one place t another one between two consecutive observations. Exploratory behavior exploring details of the field, sniffing, rearing against the walls of the field etc.

tion period locomotor activity was such that in about 90 % of the records the subject was found to have moved to another location (Fig. 2).

EXP 2.a. Location and activity at different times within a one-hour test session

The location of the untreated subject in relation to area A B C and D was calculated for each 20 min. period of a one-hour test session (film recording) During the first 20 min. the animal spent about the same amount of time in area A, B and D (Fig. 3) After 20 min. location to area C had increased The difference in time spent in location C between the first 20-minute period and the last one was significant (W $p < 0.01$) The animals were found to have changed position at least once every minute in 82 % of the records during the first 20 min period Locomotor activity declined slightly to 75 % in the last 20 min period.

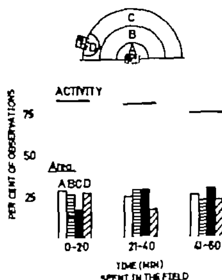


Figure 3, EXP 2a. Location and activity in the open field during a one hour test-session. The bars represent the average of 20 observations in each of 12 animals.

EXP 2.b. The effect of daily one-hour test-sessions on location and activity

Six ovariectomized females were subjected to one test-session per day for 12 consecutive days. The data from test-session 1 and 2 and data from 11 and 12 were pooled. The results are shown in Fig. 4. There were no major changes in the location profile between test-session 1 + 2 and 11 + 12. Although location in area D was less in test-session 11 + 12 than in 1 + 2 but the difference was not significant (W $p > 0.05$). Also a similar level of locomotor-activity was maintained in both test-sessions.

EXP 3

To make the technique appropriate for our purpose environmental factors that might inhibit or bias the free orientation of the animals in the field had to be controlled and eliminated as far as possible. The influence of light, sound and time during the dark period when the test-sessions were performed were tested.

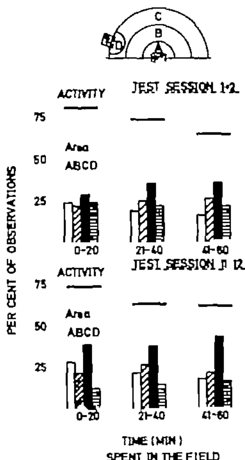


Figure 4 EXP 2.b. The effect of daily test-sessions on location and activity in the open field. The bars represent the average of 40 observations in each of 6 animals

EXP 3.a. The effect of light

During pilot experiments it was found that white light decreased locomotor activity In contrast when the white light was filtered through an infrared filter the animals moved more freely Location and activity in the field under white and infrared light conditions were compared in the following experiment

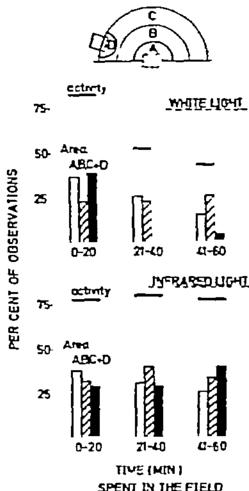


Figure 5, EXP 3a. The location and activity in the open field under white and infrared light conditions. The same subjects were run under the two light conditions. The bars represent the average of 20 observations in each of 8 animals.

PROCEDURE

Eight ovariectomized rats previously adapted to the field were subjected to one test session in white light ([illegible]5 W white light bulb replacing the ordinary 60 W bulb and the filter removed) and another one in infrared light (60 W white light bulb behind the infrared filter). The two sessions were performed 4 hours apart. No incentive animals were placed in the stimulus cages.

RESULTS

The activity and location of the animals are shown in Fig. 5 Under infrared light the animals were found to locate with roughly equal frequency in areas A, B and C+D There was a tendency however not significant (W., $p > 0.05$), to stay less in area A and more in C+D in the 41–60 min period. This feature is more obvious in the white light experiment In the 41–60 min period the animals sat significantly ($p = 0.05$) more in the peripheral area (C+D) The most obvious difference between the two light conditions was the effect on the locomotor-activity In the infrared light the subject changed position between two consecutive 1 min records in the range of 75–80 % of the three time periods. This contrasts to the white light experiment in which the activity decreased from 82 % (0–20 min) to 44 % (41–60 min.) (W $p < 0.02$).

EXP 3 b. The effect of noise

PROCEDURE

General working noise closing of doors, talking etc. was recorded on a tape recorder This noise was fed into the open field cage by a loudspeaker placed beneath the ceiling The camera was triggered 4 times/min for 60 min (Note that due to the more frequent recording the activity in this experiment cannot be directly compared with activity scores in experiments in which the camera was triggered once/min) No incentive animals were placed in the stimulus cages.

RESULTS

The effect of noise on location and activity of the animals is shown in Fig. 6 When the data obtained 10 min. before during the 5 min. of noise and 10 min. after the noise are compared a slight but not statistically significant (W $p > 0.05$), increase in location to area C+D could be seen during the period of noise The most evident effect of the noise was however a clearcut reduction of the locomotor activity in the 10 min period after the noise had been given (period 5–15 compared to 21–30 W $p < 0.01$)

EXP 3.c Test-sessions performed at different times during the dark period

PROCEDURE

Eight ovariectomized rats, kept under the ordinary 12 hour light and 12 hour dark cycle

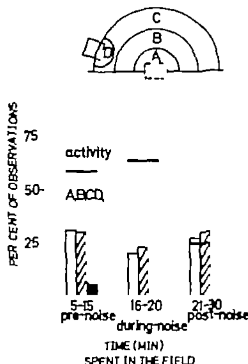


Figure 6, EXP 3.b. Th effect of noise and location and activity in the open field. The camera was triggered 4 times per min. The bars represent the average of 40 (pre and post noise scores) or 20 (during noise scores) observations in each of 12 animals.

LOCATION (AREA) and ACTIVITY	PER CENT OF OBSERVATIONS 1)	
	0 hrs	8 hr
	21	7
	31	18
CD	34	6
activity	[illegible]	[illegible]

1) 40 observations in each of [illegible]

TABLE 1 EXP 3
Location and activity in the open field [illegible] was performed [illegible] different times (0 or 8 hrs after light was turned off) within the dark period

were run either immediately after the dark period had started or eight hours after the lights were turned off. Four subjects were run at each of the two time periods. The camera was triggered once/min for 60 min. An intact male was kept in the stimulus cage in area A and an ovariectomized female in the cage in area D

RESULTS

There were no obvious differences in location or activity if the animals were run at 0 or 8 hours after the lights were turned off (Table 3)

THE INTACT FEMALE THE ESTROUS CYCLE

EXP 4

PROCEDURE

Daily vaginal smear records were taken from a group of 12 females randomly taken from the purchased batch. Only females had been kept in the same cage from the age of 22 days. These animals were not subjected to a copulation test before they were used (see General methods experimental subjects) Seven of these females were found to have a similar 5-day estrous cycle The vaginal smear records were D,D,P,E^1 (first day of cornified smear) E (second day of cornified smear). The most clearcut smears were the diestrus smears (only leucocytes) and those on the first day of cornification E^1 After 2–3 consecutive 5-day cycles each animal was run for 9 consecutive days in the open field one trial/day/animal. The cycles were not synchronized but the data were pooled with respect to the vaginal smears.

RESULTS

Figure 7 shows the percentage of records with the subject located in the vicinity of the male (area A) or female (area D) It is evident that in about 50 % of the records the female was located in the male area and in about 10 % in the female area The lowest scores in the male area were found during diestrus and the highest on the first night of cornification From a parallel study on ovulation performed in the same kind of animals it was learned that the period of LH surge in these animals was in the light period which precedes the E night Thus ovulation occurs during the dark period of E and this is the time when location in the male area is most frequent Location in the female area varied approximately inversely with the location in the male area i.e a minimum during the day of E^1

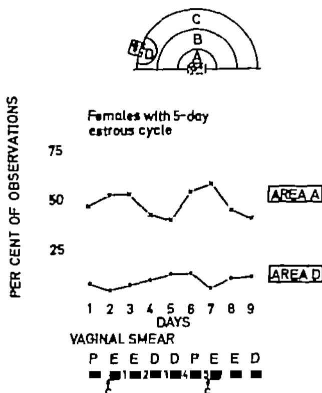


Figure 7 EXP 4 The location of intact females to the vicinity of male (area A) and an ovariectomized female (area D) during the estrous cycle. All subjects (N 7) had regular five day cycle of the D,D,P,E E type. The data were pooled with respect to the vaginal smear records. One test-session per day per animal. C the critical period (LH secretion)

THE EFFECT OF ESTRADIOL IN THE OVARIECTOMIZED RAT

EXP.s 5–10

The effect of estradiol benzoate (EB) on the location and activity in the open field was tested in seven experiments (see Table 2). In experiment 5 25 μg/kg EB was given and the effect compared to that obtained in the same subjects after oil blank treatment EXP 6 and 7 a b were conducted to study the effect of changed position of the incentive animal on the location of the EB treated female. The relationship between the dose of EB and location is studied in EXP 8 9 and 10

PROCEDURE

Experimental subjects were females ovariectomized as adults and treated according to General methods. The number of animals used in each experiment is shown in Table 2. The camera was always triggered once every minute for 60 min. EXP 5 was run for 11 consecutive days and EXP 8 9 and 10 for 8 consecutive days, one test-session per day EXP 6 was performed on day 0 1 and 4 and EXP 7 on day 0 2 and 3 after the EB injection. EXP 5 was carried out according to the cross-over design (General methods). In EXP 6 7 9 and 10 all animals had EB treatment and only one test-period per experiment was conducted. In EXP 8 the subjects were divided into 4 groups and the complete experiment consisted of 4 test-periods performed 3 weeks apart The four treatments (3 doses of EB and oil blank solution) were given at each test-period according to a Latin square arrangement so that at the end of the experiment all animals had been subjected to the 4 treatments.

Hormone-treatment incentive animals and measurements. EB and oil blank solution were given s.c on day 0 4–6 hours before the animals were run The location of incentive animals are evident from Table 2 In EXP 5 7 a 8 9 and 10 area A in Fig. 1 is considered the "male vicinity" and location in area D the "female vicinity" (except in EXP 7a in which no female incentive animal was used). In EXP 6 and 7b this is reversed The area B+C is the remaining floor space of the field. Location and activity was calculated as described in Methods.

RESULTS

EXP 5 The effect of EB 25 μg/kg
Location in the male vicinity

On days –1 and 0 the subjects were located in the male vicinity in 35–40 % of the records The initial location in the experimental and control situations were very similar (Fig. 8).

There were no difference among the 11 control (oil blank) test-sessions (Table 4 1) In contrast after 25 μg/kg EB the number of records with the female found in the male vicinity increased The effect was significant between experimental test-sessions (Table 4 4) and also between the records after hormone treatment and oil blank treatment (Table 4 7) The individual differences between the experimental and control situations for sessions 3 4,5 and 6 (day 1–4) were pooled and the average difference was 13 %.

The peak of the response location in area A, was seen on day 3 after the estradiol treatment This is further demonstrated in Fig 9 The distribution of maximal location

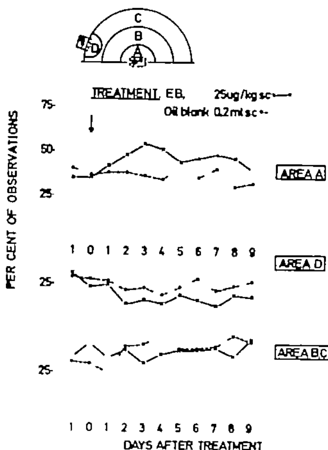


Figure 8, EXP 5. Th effect of estradiol benzoate, 25 μg/kg c. on the location of ovariectomized rats in the open field Area A incentive male vicinity Area D incentive female vicinity Treatment was given at day 0, 4 hours before the animals were run. One test-session per day per animal (N 18). Data mean values.

in the male vicinity over days is based upon the first day of the maximal score obtained during the test period (11 days). The distribution shows a peak around days 2 and 3 The figure also reveals an obvious difference in the distribution of maximal scores between the EB and oil treatment In the control situation the bell shaped distribution was not seen. In Fig 10 is shown the distribution of scores of location in the male vicinity on day 3 In the control situation the median value is 30 % After the hormone treatment there was a distribution around a median value of 50 %

DIFFERENCE TESTED	TREATMENT	LOCATION (AREA)		STATISTICAL PROCEDURE OR VALUES OF N = 18 (see line Table 1)	
Between test-sessions	Oil	A	1	A.F	NS
within the same treat-	blank	B	2		NS
ment category	0 2 ml	BC	3		NS
	EB			F	p<0 02
	25 µg/kg	D	5		p<0 01
		BC	6		NS
Between EB and oil blank		A	7	W	p<0 01
treatment (data from test-			8		p<0 01
session 3, 5 and 6 pooled)		BC	9		p<0 05

Table 4 STATISTICAL ANALYSIS EXP 5
The effect of estradiol benzoate (EB) 25 µg/kg
on location in the open field

Location in the female vicinity

The initial location was very similar in the two treatment situations. The subjects were located in the female vicinity on days −1 and 0 about 25–30 % of the time (Fig. 8). In the control situation no difference was obtained over test-sessions (Table 4.2). After the EB treatment there was a decrease in observations with the subject in area D (Fig. 8 and Table 4:5 and 4:8). This decline roughly follows the increase of location in area A. The average difference between the EB and oil treatment, data from test-session 3 4,5 and 6 pooled, was 8 %.

The main effect of the EB treatment was the change in location between the male and female vicinity: the treatment increased location in the vicinity of the male. Location in the remaining areas (B+C) changed less after the hormone treatment. In the control situation there was a slight decline in location in area D on days 2–5. There was no corresponding increase in area A but rather in area B+C.

Activity

For both treatments the activity score ranged from 70–80 %. No significant differences were obtained when the test-sessions were compared within the same treatment category or when the two treatments were compared.

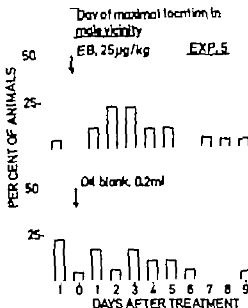


Figure 9 EXP 5. The relationship between day of treatment estradiol benzoate (EB), 25 μg/kg and day of maximal location in the male vicinity (area A) in ovariectomized rats.

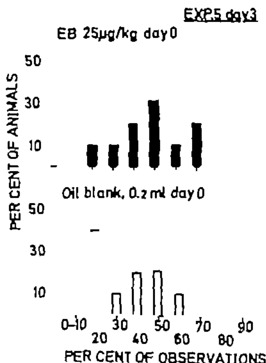


Figure 10, EXP 5 The distribution of scores on location in male vicinity in EXP 5 day 3

EXP 6 and 7 The effect of changed position of the incentive animal

These experiments were performed to investigate if the position of the incentive animal influenced the effect of the hormone treatment on the location of the experimental subject In EXP 6 the male was placed in cage X which in EXP 5 held the female and vice versa. Table 5 shows that the subject was located in the male and female vicinity to almost the same extent as in EXP 5 Thus, the specific position of the incentive animals did not influence the hormonal effect. In EXP 7 only one incentive animal was used. The other stimulus cage was empty When a sexually active male was placed in the center cage (Y) there was a significant (Sign test, p < 0.01 day 0 compared to day 3) increase in location in the male vicinity on day 3 after EB treatment (Table 6 EXP 7 a). In contrast no change of location after the EB treatment was seen on day 3 when stimulus cage Y held an ovariectomized female (Table 6 EXP 7.b)



(A) MALE VICINITY	(B) FEMALE VICINITY	(BC)	Y	(B) MALE VICINITY	(A) FEMALE VICINITY	(BC)
15	24		0	35	9	46
43	33	25	1	43	30	25
51 xx	35	14	4	50 xx	25	25

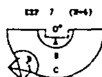
xx 25 µg/kg was given on day 0

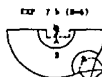
xxx) The difference between day 0 and was significant (Sign test p 0 0)

<u>TABLE 5</u> EXP 5 and 6

The ef ect of estradiol benzoate, 25 µg/kg on the location of an ovariectomized rat i the open fi ld The two expe iments diff in the position f the incentive animals

EXP 7 (N=6) EXP 7 b (N=6)





LOCATI[illegible]

(per cent of observations)

(A) MALE VICINITY	(BC)	(D)	Y	(A) FEMALE VICINITY	(BC)	(D)
42	47	11	0	50	39	11
56	32	12	2	44	40	16
60**	34	6	3	48	2	10

EB 25 µg/kg was given on day

**) The difference between day 0 and 3 was significant (Sign te[illegible] 0.01)

TABLE 6 EXP 7

The effect of estradiol benzoate 25 µg/kg [illegible] on the location of an ovariectomized [illegible] in the open field. Only one incentive animal was used (cage Y)

EXP 8, 9 and 10. The relationship between the dose of EB and location

The dose-response curve in Fig. 11 is based upon pooled scores from days 2,3,4,5 and the data calculated as a percentage of the pooled scores from day −1 and 0 (day 0 = day of treatment).

It is evident that 2.5 µg/kg EB did not influence the location of the ovariectomized female in the male vicinity (Fig. 11 and Table 7.3). A significant effect was, however obtained after 25 and 10 µg/kg EB (Fig. 11 and Table 7.1,2). The effect of 10 µg/kg was not different from that of 25 µg/kg in EXP 8 (Table 7:5).

DISCUSSION

The initial (5–10 min.) activity of the subject when placed in the open field was devoted essentially to exploring the field after which the animal oriented more towards the stimulus cages, seeking contact through the wire mesh with the incentive animals. The

the male vicinity was thus obtained roughly 2 days later i.e. day 5. This is also the time we could expect the females to mate (Young *et al.* 1941, Rodgers 1970, Hardy 1972).

It is interesting that the intact animals used in this experiment had been segregated from males when 22 days old and had never had a chance to mate. Apparently orientation to the male when in estrus needs no previous experience of direct sexual intercourse in these animals.

EB treatment

There is an evident increase in location in the male vicinity after EB treatment in the spayed rat. The increase was about 25–35 % after 10 or 25 μg/kg EB. EB 2.5 μg/kg was not effective to produce a significant effect. The peak of the response was obtained around 2–3 days after treatment.

The effect was not dependent on the position of the male in the field as the same effect was obtained if the male was located in the stimulus cage X or Y.

CHAPTER III

STUDIES ON SEXUAL MOTIVATION IN THE FEMALE RAT BY MEANS OF THE INCREASING BARRIER TECHNIQUE

In the early studies on animal drives by Moss (1924) an electric shock was used as a stimulus opposing the drive (hunger sexual and maternal) Moss worked with different amount of eclectric current and also varied quantitatively the drive incentives (e g. the period of starvation) keeping the obstruction constant This technique was adopted by Jenkins *et al.* (1926) and by Warden and Nissen (1928) who called it "the obstruction method" and used it for a more extensive study of sexual behavior in the rat The obstruction method implied that a constant current was used. The intensity of the drive was measured by the number of times the animal crossed the obstruction to reach the incentive object during a given period of time In this respect the obstruction method differs from the *increasing barrier* technique because in the latter the quantitative measurement of motivation is recorded in terms of the amount of electric current the subject is willing to tolerate in order to reach the goal

A survey of the experiments conducted is given in Table 8

METHODS

THE APPARATUS

The increasing barrier apparatus was made up of the following components (see Fig. 12) a starting cage (23x23xh.27 cm) an electric grid (23x23 cm) and a goal cage (23x46xh.27 cm). The goal cage was divided by a wire mesh into two compartments, one of which held the incentive animal The wire mesh (Fig. 12,d) was interchangeable with a wall containing a plexiglass-door or a wall with a liqued feeder The plexiglass-door could be opened by an electric motor triggered when the animal entered the goal cage To make contact with the stimulus animal the subject had to pass from the starting cage via the grid to the goal cage A wooden board (Fig. 12,a) was placed half-way across the middle of the grid to prevent the subject from jumping straight over the grid to the goal cage entrance The intensity of the grid current was increased stepwise (0 – 0.01 – 0.05 – 0.07 – 0 12 mA and thereafter by a factor of 1.3) every second time the animal crossed The time spent before the animal crossed the grid (hesitation time)

and the amount of shock the subject was willing to take was recorded by an electro-mechanical device A microswitch in the floor of the starting cage (Fig. 12,b) triggered a timer (digital counter fed by 1 pulse/sec) when a trial started. When a subject entered the goal cage (Fig 12,c) it triggered another switch located in the floor stopping one timer and starting another preset to the 15 sec interval that was the time the animal was allowed to spend in the goal cage on each trial. When a trial was over the subject was brought back to the starting cage by the observer (see flow-schedule of procedure)
grid was made of stainless steel bars 1 cm apart crossing at 90 to the direction in which the subject was running. Every second bar was interconnected and charged by a 50 cps *alternating current* The shock generator was a 340 volt transformer A 300 K resistor was connected in series with the grid to prevent a change of current due to the animal's own resistance when on the grid. The stepwise increase in the intensity of the grid current was achieved by a series of resistors coupled to a mechanical stepper which was triggered when the pre-set reinforcement time (15 sec in goal cage) was over

RECORDING OF GRID-CROSSINGS AND HESITATION TIMES

The time spent before the subject crossed the grid was termed the *Hesitation Time*. An index, H is used to indicate which trial the hesitation time is assigned to *Crossings* equal the number of times the animal crossed the grid during one test-session *When the hesitation time exceeded 5 min the session was ended. Initial pilot experi*

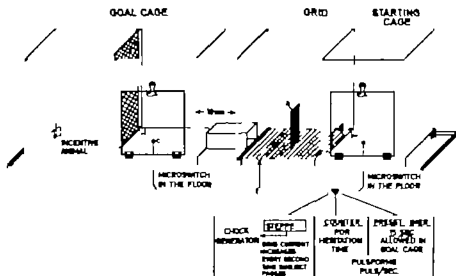


Figure 12. The *INCREASING BARRIER* apparatus. For description of details see text under 'the apparatus'

ments showed that a hesitation time of 5 min could be taken as evidence that the animal was not willing to bear additional electric current to reach the goal cage There was no inter-trial time (trial = from start until subject is in goal cage) other than the time it took the observer to bring the animal back to the starting cage If not otherwise stated data on hesitation times and crossings which appear in the figures or tables are median values

TRAINING

A Adaptation to the apparatus Before the experiments started the animals were subjected to a standardized training procedure The purpose was to habituate the animals to the apparatus. The subject was placed in the goal cage for 15 sec. and thereafter an increasing distance from the goal cage with no current fed through the grid. When the animal had learned how to reach the goal cage it was finally placed in the starting cage and 6–10 crossings over the grid permitted. These training sessions were performed, once daily for 2–3 consecutive days. After this procedure the subjects were ready for the pre-experimental training.

B Pre-experimental training After the adaptation to the apparatus or if the animals had not been run in the apparatus for more than one week, a pre-experimental training procedure was carried out The grid current was turned on and increased stepwise as in the later experimental routine Before being used in experiments the animals were run one session per day for 7–12 days or until the scores (crossings) had reached a plateau The incentive animal was the same for both the pre-experimental training and the experimental periods.

FREQUENCY OF OBSERVATIONS AND THE TIME OF DAY TEST-SESSIONS WERE CONDUCTED

If not otherwise stated, the animals were only subjected to one test-session per day Sessions were conducted during the dark period of the light regime (light from 9 p.m to 9 a m) 3–6 hours after the light had been switched off

INCREASING BARRIER FLOW-SCHEDULE OF PROCEDURE

1 Four sets f appa tus were used rranged so the 4 nimal ould be bserved imultaneously The subject we placed t andom one i each tarting cage The subjec tayed in the same appar tus throughout he same test-se ion

TRIAL f om tart until ubject is i goal cage
TEST-SESSION No f tri ls subjec i willing t pe f rm

2 Goal cage Grid Starting cage

TRIAL 1

3 Subjec crosse grid ← → U-time 3 min

TEST SESSION IS ENDED

15 sec llowed in goal cage
In er-trial ime 0
Observer b ings subject back t the tarting cage

TRIAL 2

tc

	EXP No	INCENTIVE ANIMAL G goal pe S t ti g pe	TREATMENT	NUMBER OF ANIMALS TESTED
The behavior when wat was used as reward	1	none	none	12
Crossi gs when no pe- lfi reward wa used	2	none	none	24
The trous cycl	3	G ♂	none	10
The ffect of EB		G ♂	EB 25 μg/kg or il blank	24
	5	G ♀	EB 25 μg/kg or il blank	24
	6	none	EB 25 μg/kg or oil blank	24
	7	G[1] ♂	EB 25 μg/kg or il blank	15
	8	S ♂	EB 25 g/kg oil blank	18
	9 10	♂	EB 10 μg/kg or il blank	36+18
	11 12	G ♂	EB 2 5 1 0 μg/kg or il blank	18+18
	13	G ♂	EB 0 1 μg/kg	12

[1] Direct con act llowed wi mal i goal age

♂ sexuall active mal ♀ sexually ac ive femal

G goal age S i g cage

TABLE 8 Survey f experimen on the behavior f ovari ctomi ed at i the INCREASING BARRIER apparatus

STUDEIS ON THE BEHAVIOR OF THE SUBJECTS IN THE INCREASING BARRIER APPARATUS

EXP 1

Subjects deprived of water were run in the apparatus and their willingness to cross the electric grid for water as a reward was recorded.

PROCEDURE

Ovariectomized females (N=12) subjected to the environmental conditions as stated in General Methods were deprived of water for 4 consecutive days. After the daily test the animals were given a free supply of tap water for 45 min. The apparatus was set up as described above but the wire mesh in the goal cage (Fig. 12,d) was replaced by a commercial liquid feeder When the subject entered the goal cage the feeder was triggered and 0 1 ml water was available The animals were adapted to the apparatus and then

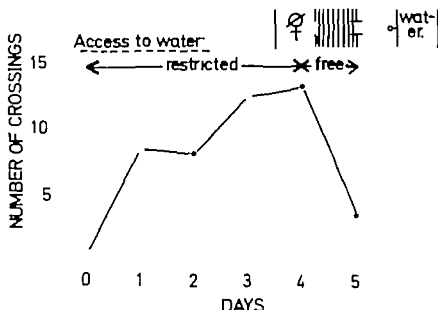


Figure 13, EXP 1 Th number of crossings made by water deprived ovariectomized rats when water was used as reward. Th number of crossings are expressed as Md-values N 12 Th procedure of water deprivation see text.

trained for one week under the same conditions as used in the experiment One test session was performed per day for 6 consecutive days. The test procedure was as described in Methods

RESULTS

Very few animals crossed the grid when not deprived of water After one day of water deprivation the number of grid crossings increased to about 8 After 4 days of restricted access to water the crossings increased to about 13 (Fig. 13 day 4). The increase in grid crossings between days 1 and 4 was significant (W $p < 0.001$) An obvious decline was seen in the willingness to cross the grid when water was again made available (Fig. 13 day 5 difference between days 4 and 5 $p < 0.01$ (W) In Fig. 14 hesitation times for different trials have been depicted in a frequency distribution diagram. When the subjects intended to cross the grid they did so essentially within 1 min on day 4 (trials 1 and 3

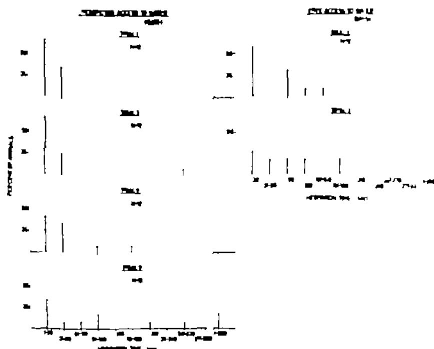


Figure 14 EXP 1 The distribution of hesitation times of ovariectomized rats for different trials when water was used as reward.

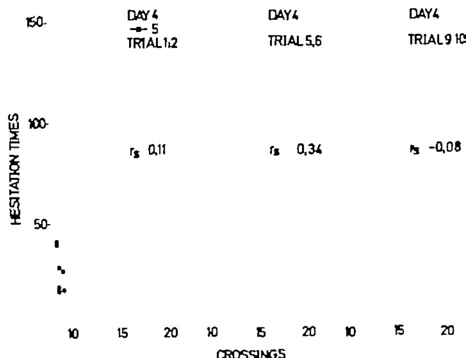


Figure 15 EXP 1 Th relationship between hesitation times (sec.) and crossings in ovariectomized rats when wate was used as reward. r_s values calculated for day 4

Fig 14) but significantly longer hesitation times were obtained on day 5 (free access to water W $p < 0.05$) There was also a significant difference between trials 1 and 3 on day 5 More time was spent in the starting cage in trial 3 than in trial 1 (W $p < 0.05$) When the access to water was restricted the animals also spent a longer time in the starting cage before crossing the grid as the number of trials increased, however this effect was not obvious until after trial 9

The relationship between grid-crossings and hesitation times was tested by the Spearman rank correlation coefficient (r_s) The average hesitation times for trials 1+2 5+6 9+10 were tested against the numbers of grid-crossings (day 4) The highest coefficient was obtained for trials 5+6 $r_s = -0.34$ This r_s value was not significantly different from zero (Fig 15)

It was concluded from this study that

1 Subjects deprived of water crossed the increasing electric barrier significantly more than when they had free access to water

Increasing the period of restricted access to water increased the willingness to cross the grid.

3 Hesitation times were longer in subjects with free access to water than in animals with restricted access to water

4 The hesitation times increased with increasing number of trials.

5 No significant correlation was obtained between the willingness to cross the grid and hesitation times for different trials.

EXP 2

Even with no incentive object in the goal cage the experimental subjects were willing to cross the grid 2 or 3 times. We have regarded this as an expression of exploratory behavior and noted that this behavior declined after several daily test-sessions. To establish the length of time until a steady state in crossings was reached the following experiment was conducted

PROCEDURE

Twenty-four ovariectomized females were adapted to the apparatus according to the Methods (Training, A) After adaptation to the apparatus the animals were given one test-session per day (except days 3 4 10 11 and 18) for 22 days. No incentive animal was in the goal cage Test-sessions were conducted and crossings recorded as described under Methods.

RESULTS

During the adaptation-period no current was fed through the grid. From day 1 the grid current was on and increased every second time the animal crossed On day 1 the average number of crossings was 6 (Fig. 16) Crossings thereafter gradually declined to a level of 2–3 crossings per session from about the 5th session (day 7) on See also EXP 6 for the effect of estradiol and oil blank treatment on grid crossing when no incentive animal was in the goal cage

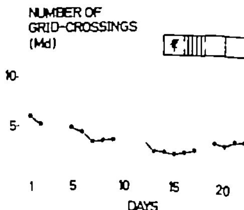


Figure 16 EXP 2. The number of crossings made by ovariectomized rats when the goal cage was empty No treatment was given. N 24

THE INTACT FEMALE, THE ESTROUS CYCLE

EXP 3

In the following experiment the willingness to cross the electric grid to reach a sexually active male was studied in intact cyclic females. In addition the effect of ovariectomy was investigated and compared to that of sham-operation

PROCEDURE

Daily vaginal smear records were taken from 10 females randomly taken from the pur chased batch. The animals had been weaned at 22 days of age and kept separate from males. In all respects they were treated as the other experimental subjects in this investigation except that they were not subjected to copulatory tests before being used in the experiment. Vaginal smear records were taken daily fro 2–3 weeks. Thereafter the subjects were ovariectomized or sham-operated Adaptation to the increasing barrier apparatus and pre-experimental training was carried out as in spayed experimental subjects Test-sessions were conducted once daily for 6 days before surgery and the animals were then rested for 6 days and again run for another 5–6 days. The incentive animal in the goal cage was a sexually active male (Fig. 17)

RESULTS

There were animals with two categories of regular cycles namely 4-day cycles (D D P E) and 5-day cycles (D D P E E^2) The day of proestrous (day for LH release) is in these animals the light period which precedes the first night of cornified epithelial cells (P/E^1) With the exception of one animal which had an irregular cycle (No 10) there was a clearcut peak in the records of crossings in these intact subjects. In 6 out of 9 animals this peak came on the first day of vaginal cornification and in the remaining animals the peak appeared one day before or one day after the first day of cornification (Fig 17) The average number of crossings for each vaginal smear was D = 7.6 P = 8.5 and for E = 11.4 There was a significant difference between the average scores for crossings with each type of smear (Fr $p < 0.001$)

After surgery the ovariectomized animals had scores at the level of the diestrous state In sham-operated subjects crossings continued to fluctuate between the presurgery di estrous and estrous scores

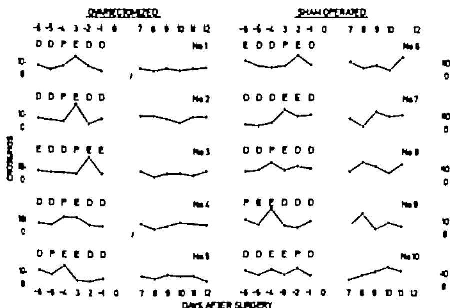


Figure 17 EXP 3. Number of crossings made by intact rats during different days of the estrous cycle and after ovariectomy or sham-operation. Surgery was performed on day 0. The goal cage held a sexually active male.

THE EFFECTS OF ESTRADIOL IN THE OVARIECTOMIZED FEMALE

EXPs 4–8

The effect of estradiol benzoate on the willingness to cross the increasing electric barrier to reach the goal box was tested in several experiments, which were analogously conducted but differed with respect to stimulus situation

PROCEDURE

Experimental subjects were ovariectomized females The number of females and type of incentive animals used in each experiment is shown in Table 8 Laboratory conditions care of animals and the experimental design were similar to those described in General Methods Procedures specific for the increasing barrier technique are described in the Methods section if not mentioned in the text below Estradiol benzoate (EB) and oil blank solution were given s c 4 hours before the test on day 0

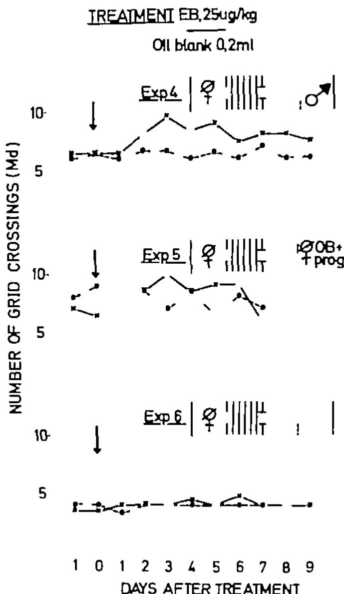


Figure 18. Th effect f estradiol benzoate (EB) on the number of crossings made by ovariectomized rats to reach contact with sexually active male (EXP 4) estrous female (EXP 5) or and empty goal cage (EXP 6).

DIFFERENCE TESTED	EXP No.	GRID-CROSSINGS C / HESITATION TIMES H		STATISTICAL PROCEDURE AND VALUES OF p (See leg Table 1)			
Between test-sessions within the same treatment category	EXP	CONTR	CT	1	F		NS
	(N 24)	EXPTLS	CT	2	F	p	0.001
	EXP 5	CONTR	C	3	F		NS
	(N 2)	EXPTLS	CT			p	0.001
	EXP 6	CONTR	CT	5			NS
	(N 2)	EXPTLS	CT	6	F		NS
Between EB and oil blank treatment (Data from test-sessions 4, 5, 6 and 7 were pooled)	EXP		CT	7	B	p	0.01
			H 1 3	8		p	0.01
			2 5				NS
	EXP 5		CT	9	B		0.05
			1 5	10	B		NS
	EXP 6		CT	11			NS
			1 5	12	B		NS
Between EXP 4 and EXP 5 (Data from test-sessions 5, 6 and 7 were pooled)							
$CONTR_{EXP\ 4} \leftrightarrow CONTR_{EXP\ 5}$				13.	[illegible]		
$EXPTLS_{EXP\ 4} \leftrightarrow EXPTLS_{EXP\ 5}$			CT	1	[illegible]		NS
$EXPTLS - CONTR_{EXP} \leftrightarrow EXPTLS - CONTR_{EXP}$					[illegible]		NS

TABLE 9 STATISTICAL ANALYSIS EXP [illegible] and 6
The [illegible] of estradiol benzoate (EB) 25 µg/kg [illegible] (EXPTLS) on grid-crossings and hesitation times performed by ovariectomized rats in the increasing barrier apparatus. Controls (CONTR) received [illegible] blank, [illegible]

RESULTS

EXP s 4, 5 and 6. Comparison of different incentives

The experiments were carried out with one test-session per day for 11 consecutive days (except in EXP 6 in which no test was performed on days 3 and 10). Fig. 18 and 19 show the effects of EB 25 µg/kg, when the incentive animal was a sexually active male (EXP 4). The number of grid-crossings increased significantly (Table 9:2 7) after the EB

variable in experimental as well as control runs. The maximal number of crossings at a test-session the subject performed during the test period is shown in Table 10 The maximal number of grid-crossings was not different in experimental and control runs in EXP 5 and 6 but was significantly higher after EB treatment in EXP 4

The distributions of hesitation times in trials 1 3 and 5 in EXP 4 are shown in Fig. 20 The data from days 2 3 4 and 5 after treatment were pooled The hesitation times in trials 1 3 and 4 but not in trials 2 and 5 were significantly shorter after EB treatment than was the case after oil injection (Table 9:8) In EXP 5 and 6 the EB treatment did not affect the hesitation times

The number of crossings and the hesitation times (in trial 3) were ranked. The ranks were based upon pooled data from days 2 3 4 and 5 The correlation between crossing and hesitation-time ranks was calculated according to the Spearman rank correlation coefficient and was found to be −0.19 Thus no correlation was obtained between the number of crossings and the hesitation times in trial 3

TREATMENT EB,25ug/kg ——
Oil blank 0,2ml- - -

Exp7.

Exp8

NUMBER OF GRID-CROSSINGS (Md)

DAYS AFTER TREATMENT

Figure 21 The effect of estradiol benzoate (EB) on the number of grid crossings made by ovariectomized rat t reach direct contact ith sexually ctive male (EXP 7) or just an empty goal cage when the starting cage held vigorou male (EXP 8)

DIFFERENCE TESTED	EXP No	GRID-CROSSINGS Cr HESITATION TIMES H		STATISTICAL PROCEDURE AND VALUES of p (See 1 Table 1)	
Between treatments within the same experiment category	EXP 7 (N 15)	CONTR	Cr	1 A F	NS
		EXPTLS	Cr	2 A Fr	p 0.001
	EXP 8 (N 10)	CONTR	Cr	3 A F	NS
		EXPTLS	C	4 F	NS
Between EB and oil blank treatment (Data from test-sessions 5 6 and 7 were pooled)	EXP 7		Cr 1 5	5 B W	p 0.01
				6 B	NS
	EXP 8		C H 1 5	7 B	NS
					NS
Between EXP 7 and EXP 8 EXPTLS CONTR ←→ EXPTLS CONTR EXP 7 EXP		day 3		9 B MWU	p 0.01
		Cr_{day} 0 1 7 7		10 B MWU	NS

TABLE 11 STATISTICAL ANALYSIS EXP 7 and 8
The effect of oestradiol benzoate (EB) 25 µg/kg s.c. (EXPTLS) on grid-crossings and hesitation times performed by ovariectomized rats in the increasing barrier apparatus Controls (CONTRL) received oil blank 0 1 ml

EXP 7 Direct contact with male in goal cage

In EXP 7 the wire mesh in the goal cage was replaced with a plexiglass door The door was automatically opened when the female entered the goal cage and direct contact with the sexually active male occurred either by the female entering the cage which held the male or by the male going into that part of the goal cage where the female stayed The latter occurred more often Two or three mounts or 15 sec with the male were allowed before the subject was brought back to the starting cage As a rule the male mounted the female even if she did not display a lordosis

After EB treatment there was a significant increase in grid crossings (Fig 21 Table 11 2,5) However the hesitation times were not different from those in the control test. The average maximal response was 14 1 crossings after the EB treatment and 13 1

crossings after the oil blank treatment The difference was not significant A comparison between EXP 4 and EXP 7 revealed that the number of crossings (difference between experimental and control tests compared) was not significantly different in the two experiments except on day 3 (Table 11:9) On day 3 the difference between experimental and control tests was greater when direct contact with the male was allowed. The difference is not very large and could to some extent be due to a slight decrease in the response of the controls on day 3 in EXP 7 On the whole the willingness to cross the grid was higher in controls in EXP 7 than in EXP 4 (see Figs. 18 and 21)

EXP 8 Male in starting cage no incentive animal in goal cage

In EXP 8 a sexually active male was placed behind wire mesh in a cage next to the starting cage The goal cage was empty (Fig. 21) There was no significant change in grid-crossings between test-sessions within the same treatment category or between treatments (EB versus oil blank, see Table 11) The number of crossings was very low compared to other experiments and slightly lower than in EXP 6 in which no incentive animal was used in the goal cage The number of crossings performed probably reflects the general level of exploratory behavior The EB treatment did not change this behavior The lack of willingness to cross the grid in the oil or untreated subject indicates that there is no obvious aversion towards the male by the spayed females.

EXP s 9–13

In the following experiments the relationship between the dose of EB and the number of grid-crossings performed to reach contact with a sexually active male was investigated

PROCEDURE

Altogether 100 ovariectomized females were used in EXP s 9–13 The number of subjects in each experiment and the specific treatment are shown in Table 8

Two test-periods with 11 (EXP 9 10) or 7 (EXP 11 12) test-sessions one per day (consecutive days except in EXP 9 in which no test was done at day 8 after EB treatment) The cross-over design was used as described in General Methods (Experimental design) was used

One test-period only was conducted in EXP 13 at which all animals were given EB treatment This experiment consisted of 7 consecutive test-sessions (one/day)

No test-period was initiated earlier than 3–4 weeks after the last treatment. EB was given 4–6 hours before test-session ? EXP s 9–13 were carried out during a period of 18 months.

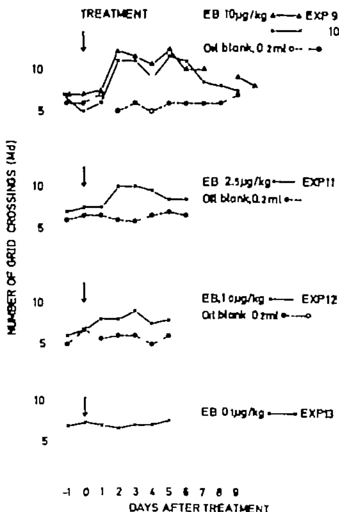


Figure 22, EXP 9 10, 11 12, 13. The effects f estradiol benzoat (EB) 0 1 1 0 2.5 and 10 µg/kg c. on the number of grid crossings made by ovariectomized rats t reach contact with sexually active male.

RESULTS

The number of grid-crossings performed after different doses of EB is shown in Fig 2_ The control data in EXP 9 and 10 were very similar and were therefore pooled. EB 10 2 5 and 1.0 but not 0 1 µg/kg significantly increased the number of crossings (Table 12) The response to EB 10 µg/kg in EXP s 9 and 10 was very similar After EB 10

DIFFERENCE TESTED	EXP No	TREATMENT EB µg/kg OIL BLANK 0 2 ml/	STATISTICAL PROCEDURE AND VALUES OF p (See also Table 1)			N
Between the sessions within the same treatment or agent	9	EB 10	1 A F	p	0 01	34
	10	EB 10	2 A F	p	0 01	18
		OIL BLANK	3 F		NS	52
	11	EB 2 5	Fr	p	0 01	18
		OIL BLANK	5 F		NS	18
	12	EB 1 0	6 F	p	0 01	18
		OIL BLANK	7 Fr		NS	18
	13	EB 0 1	8		NS	12
Between EXP 9 and 12 and EXP 10 and 12 (Data from test-sessions 4 5 were pooled)			9 MWU		0 01	
			10 MWU		0 05	

TABLE 12 STATISTICAL ANALYSIS EXP 9 10 1 12 and 13
The effect of estradiol benzoate (EB) on grid-crossings performed by ovariectomized rats i the increasing barrier apparatus

µg/kg the response reached a peak at days 2 and 3 after treatment declined at day 4 and increased again at day 5 Thereafter there was a slow decrease of the response over the following days. This bimodal feature was not seen when lower dose levels were used but it is indicated also in EXP 4 (EB 25 µg/kg)

The length of time between the EB injection and the day of maximal performance is indicated by the distribution of the days of maximal response after different doses of EB (Fig 23) The maximal response during the test period was mainly obtained around days and 3 after the EB treatment In contrast after oil blank treatment the maximal response was obtained in 44 % of the animals already at the first test-session (day –1)

The dose response after different doses of EB is depicted in Fig. 24 Data from test-sessions 4 and 5 were pooled (days 2 and 3 after treatment) and the response expressed as % increase from oil blank treatment The average number of crossings performed at test-sessions 1+ (pooled), were very similar in experimental and control test periods or between the different experiments (see figure legend of fig. 24) The number of grid-crossings the female was willing to take to reach contact with the sexual ly active male increased with increasing doses of EB The effect (at days 2+3 after

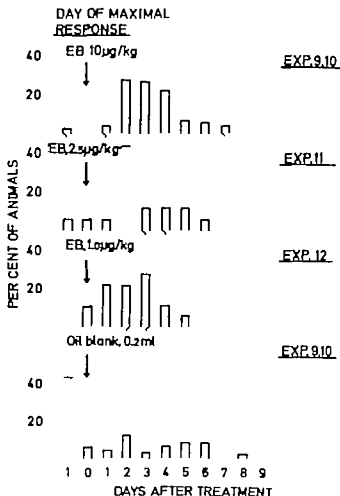
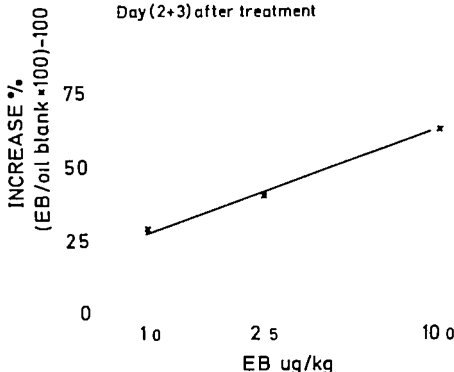


Figure 23 Frequency distribution of first day of maximal response (grid crossings) obtained after different doses of estradiol benzoate (EB)

treatment) of EB 10 2.5 and 1.0 µg/kg was different from the control data (oil blank treatment) The effect of EB, 10 µg/kg was significantly different from the effect of EB 1.0 µg/kg (Table 12 9 10)



EXP No	TREATMENT EB μg/kg s.c. OIL BLANK, 0.2 ml	GRID CROSSINGS (mean values) Days after treatment (pooled data) −1,0	2 3	Maximal No. of crossings[1]	STATISTICAL DIFFERENCE BETWEEN[2] A Days −1,0 and 2,3 B EB and OIL BLANK
9	EB 10	6 7	11.9	14.5	A,B p < 0.01
10	EB 10	6 1	11.2	13 7	A,B p < 0.01
	OIL BLANK	7.0	6 8	9.9	A NS
11	EB 2 5	7.3	10.2	12.3	A,B p < 0 01
	OIL BLANK	6 0	6.0	10.4	A NS
12	EB, 1.0	7 0	9 0	1 3	A p < 0 05 B p < 0.01
	OIL BLANK	6 1	6.5	10.3	A NS
13	EB 0 1	7.2	6 8	9 5	A NS

Maximal number f crossings performed at one test-session during test-period.
The Wilcoxon test was used

Figure 24. The relationship between the dose of estradiol benzoate (EB) and the number of grid-crossings performed by ovariectomized rats in the increasing barrier apparatus.

DISCUSSION

Number of crossings and hesitation times in water deprived animals

When water was used as a reward subjects deprived of water were willing to accept a higher grid current in order to reach the goal cage than under conditions of free access to water A longer period of restricted access to water increased the willingness to cross the grid. It is reasonable to assume that the motivation to perform in order to get water increased with increasing deprivation of water Hence the number of grid crossings the subject is willing to make reflects the degree of motivation of the animal to reach the incentive object

It is more difficult to get a quantitative estimate of motivation from the hesitation times It was true that hesitation times were longer when the subject was given free access to water than on the restricted regime but no clearcut effects were seen between different periods of water deprivation No correlation was obtained between the number of grid crossings performed and the hesitation times in different trials Motivation seems easier to measure in terms of the amount of grid current the subjects are willing to bear However hesitation times might be an important supplementary parameter to crossings. E.g. crossings without a goal-directed aim, due to restlessness or anxiety might be indicated by extreme changes in hesitation times. This information is of special interest when the effects on motivation of centrally active psychopharmacological compounds are studied

The base-level of crossings

There was evidently a certain amount of grid-crossing although no incentive object was in the goal cage (Fig. 16 and 18) The animals were willing to cross the grid 3–4 times just to explore the empty cage There were fluctuations in this response level but a very stable plateau was reached (see EXP.s 2 and 6). After the training procedure the animals regardless of the incentive situation displayed a fairly stable level of crossings (see controls EXP s 4 6 7 8) There was only one exception namely EXP 5 i.e. when a female was used as incentive animal

Th intact female

In the intact female the willingness to cross the grid to reach contact with a sexually active male was most evident at the proestrous phase of the cycle The tests were run during the dark period of the light schedule which means that the highest scores were as a rule btained during the dark period following the day of LH surge This was true in

females with 4-day as well as 5-day estrous cycles The data are in good agreement with those obtained in the open field experiments in females with 5-day cycles (see open field technique for further discussion) After ovariectomy the cyclic fluctuations in the will ingness to cross the grid disappeared which suggests a close relationship between the numbers of crossings and ovarian hormones.

Like the animals used in the open field technique these subjects had no experience of direct heterosexual intercourse Thus the increased willingness to cross the electric grid when the animal was in estrous needs no previous heterosexual copulatory experience

The incentive situation

When a sexually active male was used as incentive animal EB induced a clearcut increase in the number of crossings Also the time spent in the starting cage before crossing the grid was shorter in some of the first trials than in the corresponding trials after oil treatment No effects on crossings or hesitation times were seen after EB treatment when the goal cage was empty (EXP 6) Thus the increased amount of grid crossing after EB treatment is related to the incentive in the goal cage and not to non specific locomotor activity or exploratory behavior

When an estrous female was used as an incentive animal there was also a significant increase in crossings after EB treatment The effect was not as clearcut as when a male was used as the incentive but the difference between EXP s 4 and 5 was not significant (Table 9 13 14 15) It appears from EXP 5 that an estrous female was more of an incentive to the non-treated spayed female than was the male The number of crossings in the control situation was generally higher in EXP 5 than in EXP 4 but again the difference was not significant (Table 9 13) This could be due to the variability from test to test in EXP 5

When direct contact with the male was allowed the difference between the EB and control treatments was similar to that seen when contact was restricted by the wire mesh (EXP 4) It is surprising that controls were willing to bear a higher grid current to reach the male when direct contact was allowed than when the male sat behind the wire mesh Nor was there any tendency to avoid the male in the EXP 8 in which the female had a chance to avoid the male by crossing the grid

Effects of different doses of EB

There was a dose dependent effect on the number of crossings when different doses of EB were tested The difference between 1.0 and 10 μg/kg EB was slight but significant and both treatments were different from oil blank treatment It is obvious that in contrast to the induction of the lordosis response in the ovariectomized female the willingness to cross the electric grid was displayed although no progesterone had been given.

CHAPTER IV

STUDIES ON SEXUAL MOTIVATION IN THE FEMALE RAT BY MEANS OF THE RUNWAY-CHOICE TECHNIQUE

A variety of runway and maze techniques have been used in the investigation of animal drives. Several authors have demonstrated a relationship between food and water deprivation and the time it takes a subject to run down a runway to reach the reward (Hull 1934 Miller *et al.* 1935 Kimble 1951) These methods have also been used to study sexual drive in male and female rats (Sheffield 1951 Beach and Jordan 1956 Bolles *et al.* 1968). The rate at which an animal learns how to reach the reward in a maze or other discriminative tasks has been used as a measure of motivation Sexual drive has been measured in this way by Kagan (1955) and Whalen (1961) In the present technique a factor of discrimination was involved although the influence of learning was minimized as far as possible by preexperimental training, so that the animals could easily find their way to the incentive objects The runway-choice technique is as the name indicates, a combination of a runway and a choice situation The rationale is simply – run and choose Sexual motivation is indicated by the preferential choice in a certain situation

A survey of the experiments conducted is given in Table 14

METHODS

THE APPARATUS

A schematic description of the apparatus is depicted in Fig. 25 The main components were a runway (Fig. 25 1) a choice-cage (Fig 25) and two goal cages (Fig 25.3) The runway measured 280 x 8 x 8 cm and was covered by a plexiglass lid The lid was painted gray except for a stripe along the runway which was left transparent in order to facilitate the location of the experimental subject by the observer A 3 cm wide piece of light metal hung down in the middle of each entrance as a one-way door arrangement to preve t the animal returning to the choice cage Horizontal or vertical black lines were painted on a white background around the entrances (visual cue) The shape of the entrances was possible to alter by means of an interchangeable wooden disc with either a

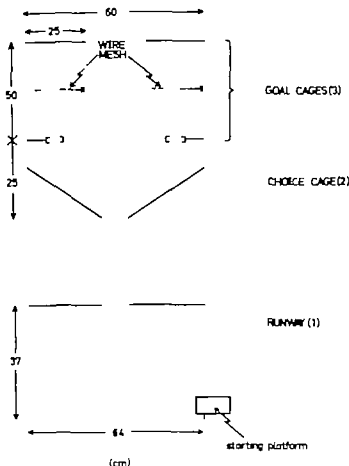


Figure 25 The RUN-WAY-CHOICE APPARATUS For description f details see text under Apparatus

circular (8 cm diameter) or a square (8 x 8 cm) hole In the goal cages the experimental subject was separated from the incentive animal by a wire mesh

Four pieces of apparatus were available Fig. 26 shows their relative position in the laboratory Two different runways were used, one in which the subject on passing through had to turn right-left-right-left (I and IV) and vice versa in the other one (II and III) The first half of each set of trials were run in apparatus I and II (or III and IV) and the remaining trials in apparatus III and IV (or I and II) When a new trial was started the subject was placed at random in one of the two pieces of apparatus.

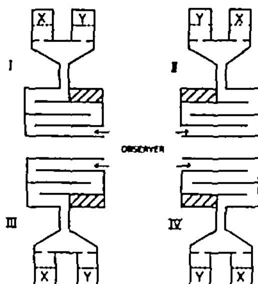


Figure 26. The arrangement of the four pieces of runway-choice apparatus in the laboratory. X and Y placements of the incentive animals.

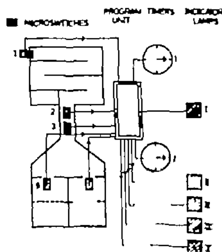


Figure 27 The runway choice apparatus: electromechanical [illegible]

THE RECORDING OF RUNNING TIME CHOICE TIME AND THE SPECIFIC CHOICE

An electromechanical device permitted recording of the time taken to run down the runway to the choice cage *Running Time* and the time spent in the choice cage *Choice Time*. The times were recorded by means of digital counters fed by 10 pulses/sec When a microswitch located in the floor of the apparatus was triggered (see Fig 27) The location of a subject at the end of a trial was shown by an indicator lamp on a light panel This information included the following items

1 if the subject stayed in the runway
  if the subject stayed in the choice cage
3 if the subject had returned from the choice cage to the runway
4 if the subject had entered one of the goal cages

A trial was ended if

a. running times exceeded *30* sec
b choice time exceeded 60 sec
c after 15 sec in a goal cage (see flow-schedule of procedure)

There was no intertrial interval other than the time it took the observer to bring the animal back to the starting position If not otherwise mentioned in the following, each experimental subject performed 20 consecutive trials on each test-session. The *Preference* for one of the goal cages was calculated either as the excess number of choices over that expected from random or as a percentage of the total number of trials the subject had entered the goal cages (for details see Table 13)

Number [illegible] f trial goal cage was chosen $_1$ and goal cage 2 $_2$

A. "Percentage of total

$$\frac{N_1}{N_2} \quad 100$$

"Excess over random

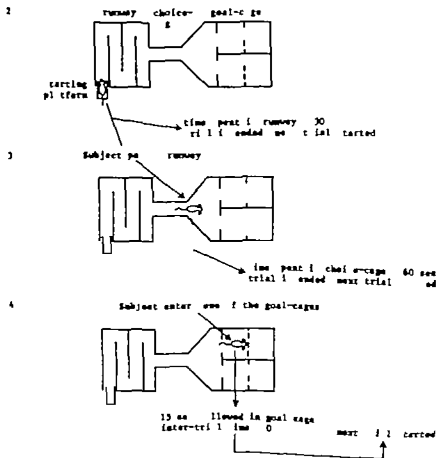
$$1 \quad \frac{N_1 \quad N_2}{2}$$

Th formula was used when the number of tri l the subjects entered the goal cages was low (N_1 N_2 10) See also procedure for each experiment

TABLE 13. CALCULATION OF THE PREFERENCE (P)

FREQUENCY OF OBSERVATIONS AND THE TIME OF THE DAY TEST-SESSIONS WERE CONDUCTED

The animals were not subjected to more than one test-session per day. The experiments were always performed at the same time of the day, namely 3–7 hours after the light had been switched off (light from 9 p.m. to 9 a.m.)



TRAINING

The experimental subjects were adapted to the apparatus and trained according to the procedure described below The experimental set-up and sort of incentive animals were always the same in the training procedure as in the subsequent experiments.

A. Adaptation to the apparatus

Day 1 and 2. The subject was placed in one of the goal cages for 15 sec. and then transfered to the other goal cage This procedure was repeated 5 times.

Day 3 and 4 The subject was allowed to explore the choice cage and to enter the goal cages. The session was ended after 10 choices or after 10 min

Day 5 and 6 The runway was now included in the procedure Each training session included 10 passages in the runway and each passage was ended either by the subject entering one of the goal cages or staying more than 60 sec. in the choice-cage

B Pre-experimental training

From day 7 on the subjects were run in accordance with the procedure used in the experiments (see Flow-schedule of procedure) However the number of trials was limited to 10 per test-session When the scores (preference for a certain incentive object) had stabilized the training was ended and the subjects were then used in experiments The duration of a complete training period was about three weeks

STATISTICAL ANALYSIS

The experimental design and the statistical treatment of the data have been described in General Methods In the runway-choice method, three parameters were recorded a) the specific choice b) the running time and c) the choice time The scores obtained fulfill the requirements of an interval scale The choice of one of the two goal cages is expressed as a preference for one of the two incentive animals (see Table 13). The individual mean values for running and choice times were normally distributed and the Student's t-test was used to test the significance of the difference between two treatments on running and choice times

	EXP No	INCENTIVE ANIMALS	TREATMENT	NUMBER OF ANIMALS TESTED
Performance of the subject in the runway choice apparatus	1	X ♂ Y ♂̸	EB 25 µg/kg or oil blank	24
	2	none	EB, 100 µg/kg or oil blank	20
	3	none	none	20
The behavior when water was measured	4	none	none	12
The effect of EB	5a b	X[1] ♂ Y ♀	EB 25 µg/kg or oil blank	24 12
	6	X[1] ♂ Y ♀	EB 10 µg/kg or oil blank	20
	7	X[1] ♂ Y ♀	EB 2.5 µg/kg or oil blank	15

[1] This arrangement i apposite cage I and II vice versa in III and IV (See Fig. 26.) ♂ sexually active male ♂̸ castrated male
♀ sexually active female

TABLE 14. Survey of experiments on the behavior of ovariectomized in the RUNWAY-CHOICE apparatus

STUDIES ON THE BEHAVIOR OF THE SUBJECTS IN THE RUNWAY-CHOICE APPARATUS

EXP 1

The relationship between the number of trials the subject entered a goal cage and the total number of trials performed and the distribution of running and choice times were studied

PROCEDURE

Ovariectomized females (N=24) were subjected to one test-session per day (= 35 trials) for 11 consecutive days. Estradiol benzoate (EB) 25 μg/kg or oil blank solution was given 4–7 hours before test-session 2. The experiments were conducted according to General Methods (cross-over design). One goal cage held an intact male and the other one a castrated male.

RESULTS

The total number of trials the subject entered a goal cage was claculated for each block of 5 consecutive trials. Data from test-session 3 through 7 were pooled. The number of trials in which the subject entered a goal cage gradually declined as the number of trials performed increased (Fig. 28 A Fr $p < 0.05$). The subjects entered the goal cages more often after EB than after the oil blank treatment but the relative decrease in incentive animal contacts with increasing numbers of trials was about the same in the two treat

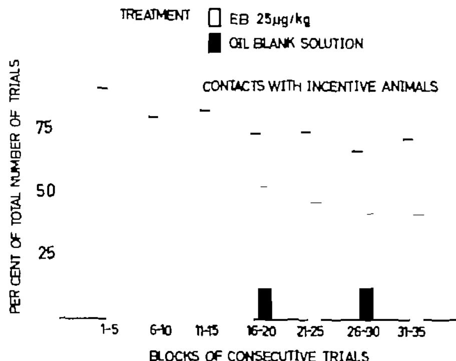


Figure 28, EXP. 1 Number of trials estradiol benzoate treated ovariectomized females entered the goal cage in the runway-choice apparatus. Total number of trials performed = 35. Data from days 2,3,4,5 and 6 after treatment were pooled. N = 24

ment categories. Within 20 trials oil treated subjects had entered the goal cages in at least half of the trials performed. This number of trials should provide a reasonable number of choices of contact with the incentive animals to allow an estimate of preference.

The distribution of running and choice times is shown in Fig. 29. The data were taken from the first 20 trials in each of the 11 consecutive test-sessions in animals treated with oil-blank at test-session 2 (more than 3000 records). The specific choice was considered. The running time was limited to 30 sec and choice time to 60 sec. It is evident from Fig. 29 that the running and choice times were less than 20 sec in about 85 percent of the trials performed when the subjects entered one of the goal cages.

The average running and choice times for each subject during the 11 test-sessions were calculated. The distribution of these mean values is shown in Fig. 30. There is a fairly normal distribution with a mean value for the total group around 1 sec regardless of which incentive animal was chosen. Running and choice time distributions were very much the same.

EXP 2

To facilitate the discrimination between the incentive objects visual cues were attached to the entrances of the goal cages. In the following, the influence of different cues on the subjects' preferences for goal cages was studied.

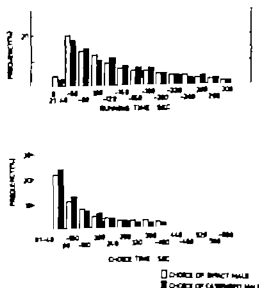


Figure 29. EXP 1. Frequency distribution of running times and choice times in oil blank treated ovariectomized rats. Data collected from 20 trials in each of 11 consecutive test-sessions. N 24

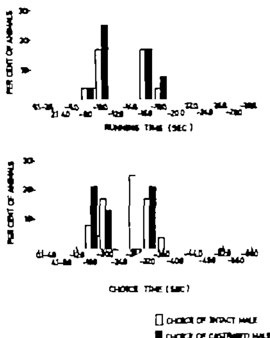


Figure 30. EXP 1 Frequency distribution of running and choice times in oil blank treated ovariectomized rats. Data from 11 test-sessions were pooled for each subject. N 24

The shape of the goal-cage entrances

PROCEDURE (A)

Ovariectomized females (N = 20) were subjected to 11 test sessions with 20 trials per test-session. The goal cages held no incentive animals. EB 100 µg/kg or oil blank solution was given on day 0 (test-session 2). A wooden disc with a circular hole was attached to the entrace of goal cage X (see Fig. 25 and Methods: the apparatus) and a disc with a square hole to the entrance of goal cage Y.

RESULTS

In Fig. 31 the preference for the circular versus the square shaped entrance is expressed as "excess over random" since the total number of times the subjects entered goal cages was small. There was a slight but consistent preference for the goal cage with the circular entrace after oil blank treatment (difference from random level W $p < 0.05$). The same preference was found after the EB treatment. The data indicate that the shape of the

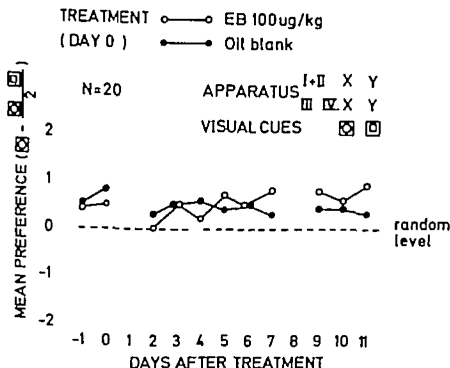


Figure 31 EXP 2. The preference for circular versus square shaped entrance after treatment with estradiol benzoat (EB) or oil blank solution at day 0

entrances might influence the choice of goal cage The preference was however not influenced by the hormone treatment The preference obtained could to some extent be due to positional factors since the goal cage with the circular entrance was always in the X-position (see Fig. 26) To study the effect of position the following experiment was performed

PROCEDURE (B)

Another 8 test-sessions were conducted with the same experimental subjects. No treatment was given The circular and squared discs were interchanged between the goal cages in apparatus I and II so that the goal cages now had the circular entrances in Y-position whereas apparatus III and IV still had the circular entrances in the X position (see Fig 26)

RESULTS

There was no difference between trials run in apparatus I and II and the preference obtained when the subjects were run in apparatus III and IV A slight but consistent preference for the goal cage with the circular entrace was still seen (W p<0.05 Fig. 32) It was concluded that the female rats used in this investigation prefered circular openings to square ones Although the hormone treatment did not influence this preference it seemed unwise to use different shapes of the goal cage entrances as a visual cue in experiments aimed to study sexual preference

EXP 3

According to Lashley (1938) rats are able to differentiate between black symbols against a white background The effect of linear symbols on the goal cage entrances on prefer ence for a goal cage was investigated.

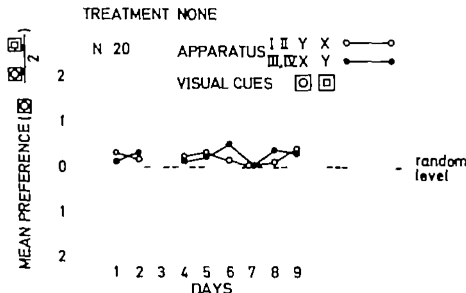


Figure 32, EXP 2. The preference for circular versus square shaped entrance in ovariectomized rats. No treatment was given. Note the difference between this experiment and the one in Fig 31 is that the circular and squared discs were interchanged between the goal cages in apparatus 1 and 2.

PROCEDURE

Ovariectomized females (N 20) were given 7 test-sessions with 20 trials per test-session No incentive animals were placed in the goal cages and no treatment was given Circular entrances to the goal cages were used. Around the entrances either horizontal or vertical black lines were painted against the white background The placement of these cues in each apparatus is evident from Fig. 33

RESULTS

No preference was obtained for one goal cage over the other one (Fig. 33) The choice of goal cage was very close to the random level in each run (W $p > 0.05$). Thus, the linear cues around the entrances could be used as indifferent cues without influencing the subjects choice of goal cage

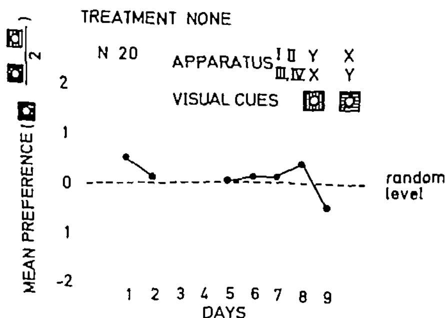


Figure 33, EXP 3 The preference for an entrance with vertical black lines versus an entrance with horizontal black lines in ovariectomized rats No treatment was given.

RESULTS

There was no difference between trials run in apparatus I and II and the preference obtained when the subjects were run in apparatus III and IV A slight but consistent preference for the goal cage with the circular entrace was still seen (W $p<0.05$ Fig. 3?) It was concluded that the female rats used in this investigation prefered circular openings to square ones Although the hormone treatment did not influence this preference it seemed unwise to use different shapes of the goal cage entrances as a visual cue in experiments aimed to study sexual preference

EXP 3

According to Lashley (1938) rats are able to differentiate between black symbols against a white background The effect of linear symbols on the goal cage entrances on preference for a goal cage was investigated.

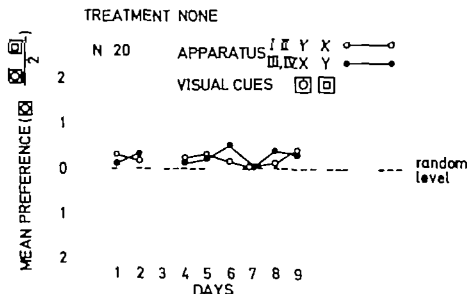


Figure 32, EXP 2. The preference for circular versus square shaped entrance in ovariectomized rat No treatment was given. Note, the difference between this experiment and the one in Fig. 31 is that the circular and squared discs were interchanged between the goal cages in apparatus 1 and 2.

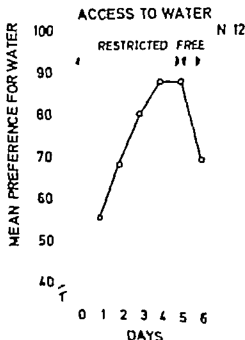


Figure 34, EXP 4 The preference for water in water deprived ovariectomized rats. For detail see procedure of Exp 4

ments were performed as described above in the Method specific to this technique Altogether 66 experimental subjects were used. The number of subjects in each experiment is shown in Table 14 A sexually active male was placed in position Y in apparatus I and II and in position X in apparatus III and IV The other goal cages held a sexually active female The visual cue for the goal cage which held the male was vertical lines and for the incentive female horizontal lines. One test-session (20 trials) was conducted the day before treatment (day 1) and in EXP 5a, 6 and 7 the day 2–7 9 and in EXP 5b day 0 through 3 after treatment. EB or oil blank solution was given on day 0 The cross-over design described in General Methods was used Treatments were given at least three weeks apart The average preference has been calculated as the choice of the male contact expressed as a per cent of the total number of trials in which contact was sought with the incentive animals (See Table 13)

RESULTS

The total number of trials the subjects chose to enter one of the goal cages was significantly higher after EB treatment (25 μg/kg) than after oil blank injection (Fig. 35 and

table). The data in EXP 5b days 1 2 and 3 were not significantly different from EXP 5a (tested by M W U test) The preference dipicted in Fig. 35 is based on pooled data from the two experiments After oil blank treatment there was a preference for the male in about 45–50 % of the trials There was no systematic change of the preference over days but a certain day to day variability EB 25 μg/kg produced a significant increase in preference for the incentive male (Table 15.1) At the day before treatment (day 1) the difference between the experimental and control test period was not significant (B W p > 0.05) The increased preference was fairly invariable from day 2 to 6 after treatment. At day 7 the preference for the male had obviously decreased The data in Fig. 35 also show that the increased preference for the male after EB treatment was mainly due to an *increased number of choices for the male incentive animal rather than a decreased* number of choices for the female (Fig 35 the table) The day of maximal preference for the male shown during the test period (EXP 5a) is depicted in the frequency distribution of Fig. 36 Most EB treated subjects had there maximal preference on days 4 5 or 6 In contrast after the oil blank treatment the day of the maximal response (preference) appeared to have a more bimodal distribution

EB 10 but not 2.5 μg/kg, produced a significant increase in the preference for the male incentive animal (Table 15.2,3) The dose-reponse after different dose levels of EB is depicted in Fig. 37 The response after 10 and 25 μg/kg was not statistically different (Table 15.4)

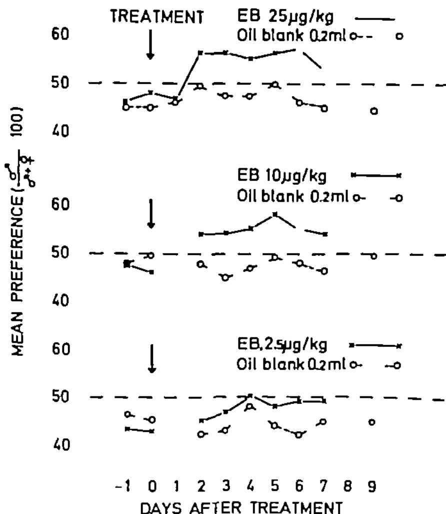
The effect of EB treatment on running and choice times are shown in Fig. 38 and 39 Data from days 2 3 4 5 and 6 EXP 5 were pooled. Running times when the choice was a male were not significantly different from trials in which the choice was a female However after the EB treatment the running times in trials in which the male was chosen were significantly lower than after oil blank treatment (Student's t-test $p < 0.01$) There were no effects on the choice times after the hormone treatment

DISCUSSION

The initial pilot-studies

A slight but consistent gradual decline in the willingness to seek contact with another animal (intact or castrated male) with an increasing number of trials was seen. After 20 consecutive trials had been performed, oil treated subjects entered the goal cage in less than half of the trials In the subsequent experiments the number of trials per test-season was limited to 10 which yielded a sufficient number of choices of incentive animals to allow a preference to be calculated

Figure 35 EXP 5 a, b, 6 7 The preference in ovariectomized rats for sexually active male versus an estrous female after estradiol benzoate (EB) or oil blank treatment. —►



TREATMENT (EXP 3a-b)	CHOICE OF	DAYS AFTER TREATMENT
		1 0 3 5 6 7 9
EB 25 μg/kg	♂	5 6 8 3 8 6 3 7 7
	♀	7 3 8 4 7 4 2 4 7 7 2
	total)	12 2 3.3 15.0 14 1 5 1 2 1 9
OIL BLANK, 0 2 ml	♂	3 8 3 3 3 3 4.8 3 6 3 3 3 3
	♀	2 3 3 2 5 5 3 9 5 6 3 5
	to)	3 0 10 9 11 1 11 1 11 9 12 1 12 1 6.8 12 3
Number of animals tested		36 12 12 36 36 36 2 24 24 2

) If ... between EB and OIL BLANK, ... EXP 3a
days 2-6 pooled 0 0 ...
days 3-4 ... p 0. 1

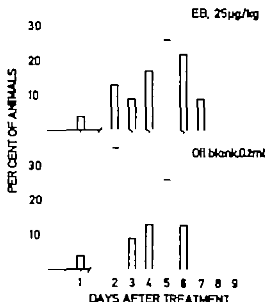


Figure 36, EXP 5 Frequency distribution of first day of maximal response (preference for a sexually active male versus an estrous female) in ovariectomized rat treated with estradiol benzoate (EB) at day 0

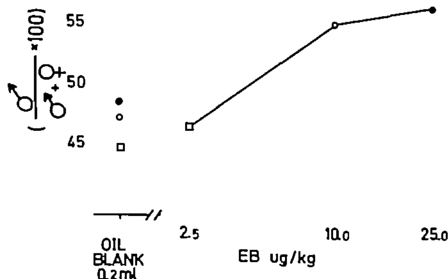


Figure 37 EXP 5 a, 6, 7 The relationship between the dose of EB and the preference for sexually active male versus an estrous female in ovariectomized rats. Treatment was given at day 0. Data from test-sessions 2, 3 4 5 and 6 after treatment were pooled. Controls received oil blank solution 0.2 ml.

DIFFERENCE TESTED	EXP No	TREATMENT	STATISTICAL PROCEDURE AND VALUES OF p (See ... Table 1)				
Between test-sessions	5a	EB 25 µg/kg	1	F	p	0 01	2
within the same treatment	b	EB 25 µg/kg	2		p	0 01	12
category	5a	Oil blank 0 2 l	3		NS		24
	b	Oil blank 0 2 ml			NS		12
Between EB and oil blank	5a	EB 25 µg/kg	5	U	p	0 01	24
treatment Data from days	6	10	6		p	0 01	20
2-6 pooled	7	2 5	7		NS		15
Between EXP 5 and 6			8		NS		
Data from days 2-6 pooled							

TABLE 13 STATISTICAL ANALYSIS EXP 5 6 and 7
The effect of oestradiol benzoate (EB) on the preference for sexually active male versus an oestrous female performed by ovariectomised rats in the runway-choice apparatus

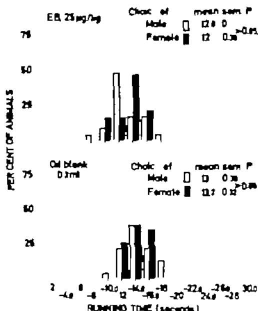


Figure 38, EXP 5 a. Frequency distribution of running times after estradiol benzoate (EB) or oil blank treatment. Data from test-sessions of day 2, 3, 4 5 and 6 after treatment were pooled. N 23.

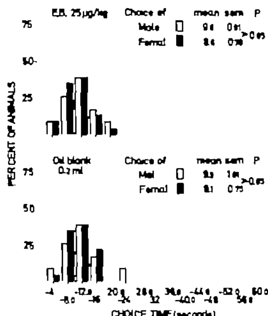


Figure 39 EXP 5 a. Frequency distribution f choice times after estradiol benzoate (EB) or oil blank treatment Data from test-sessions day 2 3 4 5 nd 6 fter treatment were pooled. N 23

Visual spatial and olfactory cues were probably used before the subject entered one of the goal cages The relative importance of different cues in this situation is difficult to predict However in order to facilitate the discrimination visual cues were applied to the entrances of the goal cages Two kinds of visual cues were tested painted lines and different shapes of the entrances Since the choice of empty goal cage was not randomly distributed when circular versus square entrances were used different shapes of the entrances were not utilized in the following experiments It has been demonstrated that rats can easily distinguish between horizontal and vertical black lines (Lashely 1938) The present data showed that the choice of goal cage was not influenced by this kind of visual cue

The experiment conducted in which water was used as a reward demonstrates that the subject easily learned to find the goal cage which held the reward The measured response the preference could take values up to at least 90 %

The effect of EB treatment

When a sexually active male and an estrous female were used as incentive animals oil blank treated subjects had a preference for the male In the majority of test-sessions, in

less than 50 % of the trials in which a goal cages was entered After EB treatment (25 and *10 μg/kg) there was a clearcut increase in preference for the male which was consistent* for several days. The maximal response obtained during the test period was most frequently seen 4–6 days after the EB treatment

The choice time was not changed by the hormone treatment As to running times no difference was found between trials in which a male was chosen and trials in which contact with a female was sought. This is in agreement with the data of Bolles *et al.* (1968) There was however shorter running times after EB than after oil blank treatment, this effect was significant only when the male was chosen Running times and choice times seems to be less suitable parameters than preference in measureing the sexual motivation However in a study of the effects of drugs on sexual motivation these parameters might give complementary information about changes in locomotor activity

SUMMARY AND GENERAL CONCLUSIONS

Different techniques to measure the sexual drive and the effects of estradiol benzoate on the sexual motivation in the ovariectomized rat were studied

Three techniqeus were employed to study the urge of the female rat to seek contact with a sexually active male The methods differed from each other with respect to the behavior the subject had to perform to reach this contact. The contact between the experimental subject and the incentive animal was in most experiments restricted by a wire-mesh screen preventing direct sexual intercourse

The *OPEN FIELD* method measured the orientation of the female towards a male or a female How much of a negative stimulus (crossing an electric grid) the female was willing to take to reach contact with the male was recorded in the *INCREASING BARRIER* technique The *RUNWAY-CHOICE* method was designed to investigate the preference for a certain incentive animal in a run and choose situation It seemed desirable that measurements of as complex a matter as sexual motivation should involve parallel recordings with more than one technique In addition each of the present techniques involve parameters which will reflect non-specific changes of e.g locomotor activity (hesitation times in the increasing barrier change of position in the open field, running times in the runway-choice method)

Estradiol benzoate (EB) induced an increased location to the male and decreased location to the female vicinity in the open field Significantly more grid current was endured to reach contact with a sexually active male after EB treatment and the hormone produced a clearcut increase in preference for a sexually active male over an estrous female measured with the runway-choice technique

When the data from the three techniques are taken together it is suggested that EB induced in the ovariectomized rat an urge to actively seek contact with a sexually vigorous male There was a consistent finding that the male was a more effective incentive than a female Experiments in which different stimulus situations and incentive animals were used suggest that the predominant incentive to the drive to seek contact with the male was sexual rather than social For the same reason it is unlikely that the hormone activated response was simply an expression of increased exploratory or locomotor performance

To induce a significant effect EB 10 μg/kg s.c was effective in all three methods In

the increasing barrier technique even 1.0 μg/kg was sufficient The hormone-induced effect was evident after two days but the maximal response appeared later in the runway choice method (4–6 days) than in the open field and increasing barrier techniques (2–3 days). In contrast to estrogen + progesteron-activated copulatory behavior in the female rat, estrogen treatment alone was effective in producing the sexual motivation The dose of EB necessary to produce sexual motivation was similar to the one required to induce lordotic behavior (Meyerson 1964).

The rather narrow deflection of the EB induced response between non-significant and maximal response and also the variability in the response limit the quantitative estimates within the dose-response interval of the estrogen treatment This fact does not exclude the possibility that the response measured with the present techniques could increase above the effect obtained by a maximal hormone treatment

There is evidently a broad neuroendocrine and psychopharmacological field within which the present methods could be applied. Preliminary data are available as to the effect of testosterone treatment on sexual motivation the hormone given either neonat ally or to the adult animal (Meyerson and Lindström, 1971b c, d, Meyerson Nordström and Ågmo 1971)

ACKNOWLEDGEMENTS

The authors would like to extend their sincere gratitude to

Professor E Bárány for encouraging us to start and his continuous interest in the project Mr Claes-Iwar Sjöberg and Mr Jan-Erik Hägglund for invaluable help in constructing the equipment and particularly we are indebted to Miss Ann-Kristin Söderlund and Miss Marita Berg for their patience in collecting the behavior data and Mr Alf Hallberg for his care of the animals Illustrations were made by Miss Marita Berg.

Our thanks are also due to Mrs Ann-Mari Kjellberg, Mrs Mona Nilsson and Miss Monica Nilsson for preparation of the manuscript and Mr Manne Fredriksson for photographic details

The computer programs were skilfully made by Ulla Hedqvist Uppsala Computer Center and we also thank Mr B Hallenberg for statistical consultants at the early stage of the investigation.

For constructive criticism, revision of the language and a cheerful working atmosphere we also thank visitors and members of the psycho-endocrine group Asta Palis, Mona Eliasson, Erna-Britt Nordstrom, Peter McDonald, Carl-Olof Malmnäs Anders Ågmo

The estradiol benzoate and progesterone was kindly supplied by Organon through Erco Stockholm.

The investigation was supported by NIH grant RO1-HD4108-03 and Swedish Medical REsearch Council grant 14X-64-08

REFERENCES

BEACH, F.A. and L. JORDAN, Effect of sexual reinforcement upon the performance of male rat in straight runway *J Comp. Physiol.* 1956 *49* 105–110

BERMONT G., Response latencies of female rats during sexual intercourse. *Science* 1961 *133* 1771–1773

BERMONT G. and W.H. WESTBROOK Peripheral factors in the regulation of sexual contact by female rats. *J Comp. Physiol. Psychol.* 1966 *61* 244 250

BOLLES, R.C., H.M. RAPP and G.C. WHITE Failure of sexual activity to reinforce female rats. *J Comp. Physiol. Psychol.* 1960 *65* 311–313

BROODHURST P.L., Determinants of emotionality in the rat. *Brit J Psychol.* 1958 *49* 12–20

CANDLAND D.K. and B.A. CAMPBELL, Development of fear in the rat as measured by behavior in the open field. *J Comp. Physiol. Psychol.* 1962. *55* 593 596

HARDY D F Sexual behavior in continuously cycling rats. *Behaviour* 1972. *41* 288–297

HARDY D F and J.F DeBOLD Effects of coital stimulation upon behavior of the female rat. *J Comp. Physiol. Psychol.* 1972 *67* 400–408

HULL, C.L., The rat speed-locomotion gradient in the approach to food. *J Comp. Psychol.* 1934 *17* 393–422.

JENKINS, M The effect of segregation on the sex behavior of the white rat as measured by the obstruction method *Genet. Psychol. Monogr* 1928 *3* 455–571

JENKINS, T.N., L.H. WARNER and L.J WARDEN Standard apparatus for the study of animal motivation. *J Comp. Psychol.* 1926 *6*, 361–382

KAGAN J Differential reward value of incomplete and complete sexual behavior *J Comp. Physiol Psychol.* 1955 *48* 59–64

KIMBLE, G.A., Behavior strength as a function of the intensity of the hunger drive. *J Exp. Psychol.* 1951 *41* 341–348

LASHLEY K.S The mechanism of vision XV preliminary studies of the rat's capacity for detail vision. *J Gen. Psychol.* 1938 *18*, 123–193

MEYERSON, B.J The effect of neuropharmacological agents on hormone-activated estrus behaviour in ovariectomized rats. *Arch int. Pharmacodyn.* 1964 *150* 4–33

MEYERSON B.J and L. LINDSTRÖM, Sexual motivation in the estrogen treated ovariectomized rat. Third Internat. Congr on Hormonal Steroids, Symposium lecture Hamburg 1970

MEYERSON B.J Sexual motivation in the neonatally androgen treated female rat. Psychoneuroendocrine Congress, Budapest 1971

MEYERSON, B.J and L LINDSTRÖM Sexual motivation in the neonatally androgen treated female rat. *Acta pharmacol toxicol.* 1971 *29* suppl. 4

MEYERSON B.J E. NORDSTRÖM and A ÅGMO Sexual behaviour and testosterone in the female rat. *Acta pharmacol. toxicol.* 1971 *29* Suppl 4

MILLER, N.E. and W.R. MILES, Effect of caffeine on running speed of hungry satisfied, and frustrated rats. J Comp Psychol 1935 *20*, 397–412

MOSS, F A., A study of animal drives. J exp Psychol. 1924 7 165–185

NISSEN H W The effects of gonadectomy vasotomy and injections of placental and orchic extracts on the behavior of the white rat *Genet. Psychol Monogr* 1929 *5* 455–547

PEIRCE, J and NUTTALL, L., Self-paced sexual behavior in the female rat J Comp Physiol Psychol. 1961 *54* 310–313

RODGERS C.H., Timing of sexual behavior in the female rat. *Endocrinology* 1970 *86*, 1181–1183

RODGERS C.H and O.T LAW The effects of habenular and medial forebrain bundle lesions on sexual behavior in female rats. *Psychonom. Sci.* 1967 *8*, 1 2

SCHWARTZ, N.B., A model for the regulation of ovulation in the rat *Recent Progress in Hormone Research* 1969 *25* 1–55

SIEGEL, I S Nonparametric statistics for the behavioral sciences, London, McGraw Hill 1956

SHEFFIELD F J WULFF and R. BACKER, Reward value of copulation without sex drive reduction. *J Comp. Physiol. Psychol.* 1951 *44* 3–8

WARNER, L.H., A study of sex behavior in the white rat by means of the obstruction method. *Comp. Psychol. Monogr.* 1927 *4* 1–58
WARDEN L.J and H.W NISSEN An experimental analysis of the obstruction method f measuring animal drives. *J Comp. Psychol.* 1928 *8*, 325–342.
WHALEN R.E., Effects of mounting without intromission and intromission without ejaculation on sexual behavior and maze learning. *J Comp. Physiol. Psychol.* 1961 *54* 409–415
YOUNG W.C., The hormones and mating behavior In: Sex and Internal SEcretions, William and Wilkins Co Baltimore Md 1961 p. 1173
YOUNG, W.C., J.L. BOLING and R.J BLANDAU The vaginal smear picture, sexual receptivity and time of ovulation in the albino rat. *Anat. Rec.* 1941 *80* 37–45
ZARROW M.X. YOCHIM, J.M. and J.L. McCARTY Experimental endocrinology Academic Press, London, 1964

UPPSALA OFFSET CENTER ab 1973

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 390

STUDIES ON THE INHIBITORY NERVOUS CONTROL OF GASTRIC MOTILITY

BY
HASSE ABRAHAMSSON

Göteborg 1973

UPPSALA OFFSET CENTER ab [illegible]

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 390

STUDIES ON THE INHIBITORY NERVOUS CONTROL OF GASTRIC MOTILITY

BY
HASSE ABRAHAMSSON

Göteborg 1973

ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 390

From the Department of Physiology
University of Göteborg
Göteborg Sweden

STUDIES ON THE INHIBITORY NERVOUS CONTROL OF GASTRIC MOTILITY

BY
HASSE ABRAHAMSSON

Göteborg 1973

CONTENTS

This summary is mainly based on studies reported in the following papers

I Abrahamsson H and G Jansson Vago-vagal gastro-gastric relaxation in the cat Acta physiol scand 1973 In press

II Abrahamsson, H Vagal relaxation of the stomach induced from the gastric antrum Acta physiol scand 1973 In press

III Abrahamsson, H Reflex adrenergic inhibition of gastric motility elicited from the gastric antrum Acta physiol scand 1973 In press

IV Abrahamsson, H G Jansson and J Martinson Vagal relaxation of the stomach induced by apomorphine in the cat Acta physiol scand 1973 In press

V Abrahamsson, H and P Thorén Vomiting and reflex vagal relaxation of the stomach elicited from heart receptors in the cat Acta physiol scand 1973 In press

These papers are referred to in the text by their Roman numerals

CONTENTS

INTPODUCTION

Gastric motor functions

The motor function of the stomach is to act as a reservoir to mix and grind ingested food, and to gradually empty its content in suitable portions and rate to the intestine While an amount of studies have dealt with the gastric movements responsible for the latter two functions rather little work has been performed concerning the process of gastric filling and storage and the control mechanisms involved in these pro cesses

Gastric reservoir function Already Cannon (1898) stated that the car diac part of the stomach acts as a reservoir pressing out its contents a little at a time as the antral mechanism is ready to receive them Other early observers (e g Kelling 1903 Grey 1918) noted that the stomach can be greatly filled with only slight increases of intragastric pressure Gianturco (1934) carefully mapped out by an X-ray method that it is the fundus and corpus parts that subserve the gastric reservoir function showing very little pressure rise in response to filling in contrast to the gastric antrum Cannon and Lieb (1911) found that during deglutition the stomach relaxes already when food passes the pharynx and the upper part of esophagus and termed this response gastric receptive relaxation (cf Lind et al 1961) Though little was known about the mechanisms involved at this time Grey (1918) observing the pronounced capacity of the stomach to accomodate to increased volumes included in this term also the adjustments of the gastric wall in re sponse to direct intragastric filling

Profound relaxation of the proximal part of the stomach also occurs during vomiting as an early gastric change during this act (Openchowski 1889, Cannon 1898 Hesse 1913) This gastric motor event is also a receptive relaxation contributing to a reversal of the usual gastro-duodenal pressure gradient (Davenport 1971 p 79) It is followed by complex cyclic movements of the gastric antrum and the duodenum (Smith and Brizzee 1961) probably including antiperistaltic duodenal movements (Weisbrodt and Christensen 1972) These move ments lead to a transport of gastric and intestinal contents to the al ready relaxed corpus-fundus part where it accumulates until expulsion of vomitus occur due to spasmodic contractions of abdominal skeletal muscles The behaviour of the stomach during emesis seems to be the same whether vomiting occurs spontaneously (Barclay 1936 Lumsden and Holden 1969) is induced via autonomic nervous afferents (Cannon 1911) acting on the medullary vomiting centre or is induced by the

centrally acting drug apomorphine (Cannon 1898 Hesse 1913) This drug acts on the chemoreceptor trigger zone in area postrema, from which the vomiting center located deeper in the medulla, is then activated (Wang and Borison 1952)

The myogenic and nervous control mechanisms responsible for the regulation of the gastric reservoir part are still largely unknown Recently, tonic electrical oscillations, with a frequency about 1-1 5/sec and perhaps triggering the development of mechanical tension have been observed in the gastric corpus-fundus muscles In the guinea-pig fundus muscles (Golenhofen et al 1970) this electrical activity was found to be myogenic in nature In the gastric corpus region of conscious dogs (Arimoro et al 1970) this activity was suppressed by esophageal as well as gastric distension both procedures known to elicit gastric receptive relaxation (Cannon and Lieb 1911, Grey 1918)

The role of extrinsic nerves in the regulation of gastric reservoir function has been only sparsely studied However after vagotomy in man symtoms of impaired gastric reservoir capacity such as epigastric fulness and early satiety are common (Aune 1969) These disturbances correspond well to the finding that vagotomy leads to an increased gastric tone observed in recent gastric pressure-volume studies in man (Aune 1969 Köster and Madsen 1970 Stadaas and Aune 1970) and dogs (Carter et al 1972) Thus there are definite suggestions that besides an intrinsic gastric motor control the participation of extrinsic nerves are also essential for an adequate gastric reservoir function although the details of this extrinsic nervous control have remained largely unknown

Gastric mixing and emptying These two functions will be only briefly discussed here They are generally looked upon as being to a large extent dependent on the same basic gastric motor phenomenon, the gastric peristaltic wave (see Code and Carlson 1968) The peristaltic waves can vary in appearance but they usually originate in the oral part of the corpus and travelling downwards as a band of contraction to the antrum, where they often end with a simultaneous contraction of the terminal antrum and the pyloric canal While smaller waves propel very little content but provide some mixing the more vigorous peristaltic contractions provide both mixing and propelling of the contents As the peristaltic contractions move over the corpus towards the antrum the orifice of the contracting ring is initially relatively large so that the gastric contents can retropel back towards the corpus With diminishing orifice of the propagating contraction ring towards the antrum the propulsion of contents becomes more pronounced Even during the terminal antral contraction both propelling and retropelling occur

For further details of these gastric motor functions and their control, see Code and Carlson (1968), Daniel and Irwin (1968) Edwards and

Rowlands (1968) Kelly and Code (1971) Bedi et al (1972)

The extrinsic nerve supply to the stomach

Vagal cholinergic excitatory fibres It has long been known that the stomach smooth muscles receive an excitatory cholinergic nerve supply by the vagi; these fibres will be only briefly outlined in this context Their preganglionic fibres run in the vagi, making contact with the postganglionic neurons that are located in the mural myenteric plexa (Fig 1 p 21) These neurons in turn innervate the smooth muscle cells and release the excitatory transmitter acetylcholine to the effector cell There are also some cholinergic excitatory fibres present in the splanchnic nerve supply to the stomach but their functional significance is, however, as yet obscure The importance of the extrinsic nerves in the control of gastric motility is suggested by the observation that there are 6 times as many synaptic connections within the gastric myenteric plexus as within that of the small intestine and that vagotomy leads to a profound reduction in the number of synapses (Gabella 1970)

Vagal non-adrenergic relaxatory fibres The nature of the extrinsic inhibitory nervous control of the stomach as well as that of the intestine was for a long time an enigma which first in the last decade has begun to be clarified Although the presence of inhibitory vagal fibres to the stomach was discussed already at the turn of the century (Langley 1898 Kelling 1903) the nature of this inhibition was for a long time obscure The concept was (McSwiney 1931) that vagal stimulation produced gastric inhibition when prevailing gastric tone was high and contraction when gastric tone was low The quantitative effect of the vagal inhibitory fibres was considered to be slight (see Thomas 1957) and they were thought to be adrenergic in nature (Greeff et al 1962 Paton and Vane 1963)

This picture has later on been markedly changed First Martinson who clearly revealed the existence of distinct groups of excitatory and inhibitory vagal fibres (Martinson and Muren 1963 Martinson 1964) showed that the transmission mechanism for the vagal inhibitory fibres was not an adrenergic one or involved any other known transmittor substance (Martinson 1965) and Campbell (1966) came to the same conclusion The effect of ganglionic blockade suggested preganglionic fibres to be present in the vagal nerves and postganglionic neurons in the gastric wall Second Jansson and Martinson (1965) showed that these vagal fibres produce profound relaxation of the corpus-fundus

part of the stomach, capable to manyfold the gastric volume thus really controlling the capacity of the reservoir The characteristics of the gastric response to activation of this vagal supply, being in many respects very different from the inhibitory effect of adrenergic fibres (see Jansson 1969 b), have led to the term relaxatory fibres which will be used in the following account

This relaxatory vagal fibre supply was by Jansson (1968, 1969 a) and Ohga et al (1969 1970) shown to be the efferent link to the stomach in vago-vagal reflexes elicited by electric afferent vagal stimulation The role of these vagal fibres in the gastric receptive relaxation during deglutition was indicated by the findings (Abrahamsson and Jansson 1969) that esophageal distension mechanical pharyngeal stimulation and swallowings caused profound gastric relaxation by reflex activation of these vagal fibres

Intramural non-adrenergic inhibitory neurons have now been found in many parts of the digestive tract but appear to be usually devoid of preganglionic extrinsic fibres except in the stomach The transmittor substance is thought to cause hyperpolarisation of the smooth muscle cell by producing a specific increase in K^+ conductance Inhibitory junction potentials have been recorded in gastric smooth muscle cells in response to vagal stimulation (Beani et al 1971) Evidence has recently been presented (Burnstock et al 1970, Burnstock 1972) that the non-adrenergic inhibitory transmittor substance might be adenosine triphosphate or a related nucleotide For details of the events at the effector cell see review by Burnstock (1972)

Adrenergic fibres to the stomach Most of the adrenergic fibres to the stomach are derived from the splanchnic nerves with the postganglionic neuron located in the celiac ganglion The earlier concept was that adrenergic inhibitory fibres, like the excitatory fibres from the vagi, innervate the gastrointestinal smooth muscle directly whereby the two sets of fibres should subserve a reciprocal control over gastrointestinal movements (Bayliss and Starling 1899) However experiments in acutely vagotomized preparations showed that rather high stimulation frequencies of the splanchnic nerves were required to produce clearcut inhibition of gastric motility (McSwiney and Wadge 1928, Martinson 1965 Campbell 1966) The same is true concerning the intestine (Celander 1959) suggesting an indirect inhibitory mechanism such as overflow of transmittor substance from structures at a distance from the gastrointestinal smooth muscle cells (cf Beani et al 1971)

Histochemical studies (Norberg 1964 Jacobowitz 1965, see also Burnstock 1972) showed the existence of adrenergic ramifications close to the intramural ganglion cells observed also in man (Baumgarten 1967) Gabella (1971) observed typical synaptic clefts between adrenergic varicosities and intramural ganglion cells in the intestine

Physiological evidence for an inhibitory effect of adrenergic fibres on gastric motility, but exerting its effect on the intramural neurons, was presented by Jansson and Martinson (1966) and Jansson (1969 b) Gastric contractile activity, induced by efferent vagal stimulation and thus due to activity in intramural excitatory neurons could be markedly inhibited by stimulation of splanchnic adrenergic fibres already at quite low stimulation frequencies On the other hand when there was no prevailing activity in cholinergic neurons, activation of adrenergic fibres to the stomach had no or only a very scant inhibitory effect In agreement with motility studies in the intestine (Kewenter 1965) Paton and Vizi (1969) found that the amount of acetylcholine liberated from nerves in the gut is markedly reduced when α-adrenergic receptors are stimulated by noradrenaline All these results support the concept of a ganglionic site of action for adrenergic fibres in regulation of gastro-intestinal motility

It is well established that the splanchnic nervous activity inhibits gastric motility during various pathophysiological conditions e g due to nociceptive stimuli in the gastrointestinal tract (see Thomas and Baldwin 1968) A more strictly physiological role of splanchnic adrenergic fibres to the stomach was suggested by Jansson and Martinson (1966) They found that the spinal intestino-gastric inhibitory reflex could be elicited by such slight degrees of intestinal distension that it must be well within a physiological range To what extent activation of receptors in the stomach itself influence gastric motility reflexly via the splanchnic nerves has not been established

Afferent fibres and receptors Afferent nerve fibres from the stomach run in both vagal and splanchnic nerves It is noteworthy that most fibres are afferent (sensory) both in the abdominal vagus (Daly and Evans 1953 Agostoni et al 1957) and in the splanchnic nerves (Foley 1948) Precise information about the properties of gastro-intestinal receptors requires recording from the afferent fibres with single fibre techniques (Paintal 1954 Iggo 1955) As far as gastric receptors are concerned several such investigation has been performed on vagal afferents but very few on afferents in the splanchnic nerves Studies on vagal afferents have revealed two distinct categories of gastric mechanoreceptor slowly adapting mechanoreceptors and rapidly adapting mechanoreceptors with chemoreceptor properties as well

The slowly adapting mechanoreceptors as studied in cats goats and sheep (Paintal 1954 Iggo 1955 and 1957 a Leek 1969 Mei 1970 Harding and Leek 1972 a), are activated both by passive distension of the viscus and by isometric contraction in the receptor area They are therefore termed tension receptors considered as being functionally coupled in series with the smooth muscle cells They give a well sustained response when an applied distension is kept steady Receptors

with similar properties and with vagal afferents have also been found in the thoracic esophagus the lower esophageal sphincter (Mei 1970 Mei et al 1972) and in the intestine (Iggo 1957 a Mei 1970, Harding and Leek 1972 a)

Rapidly adapting mechanoreceptors located in or near the gastric mucosa have been studied in cats and ruminants (Iggo 1957 b Davison 1972 Harding and Leek 1972 a, b Leek 1972) They are activated particularly by brushing the mucosa and give only transient on - off responses when exposed to a moderate steady stretch Recent studies by Harding and Leek (1972 a, b) show that one and the same such receptor unit can also respond to acid and alkalic solutions

Gastric receptors with afferents in the splanchnic nerves have only recently been investigated with electrophysiological techniques Reflex effects of abomasal distension on the motility in the ruminant reticulum (Titchen 1958) suggest that such afferents emanating from gastric mechanoreceptors, exist and seem to be involved in the regulation of gastrointestinal motility Single fibre investigations on splanchnic nerve afferents (Morrison 1972) showed mechanoreceptors in the mesentery and beneath the intestinal serosa giving a rapidly adapting discharge to distension of the intestine but a maintained response when the mesentery was stretched Gastric receptors with splanchnic nerve afferents and with a similar localization as the mentioned intestinal receptors have recently been found to give a slowly adapting discharge to antral distension and can be activated also by antral contractions (Morrison personal communication)

AIM OF THE PRESENT STUDIES

Earlier studies on the role of the vagal non-adrenergic relaxatory fibres in reflex regulation of gastric motility (Jansson 1969 a Abrahamsson and Jansson 1969) have strongly suggested that these fibres mediate gastric receptive relaxation during deglutition Thus they are evidently of importance in the control of various gastric motor events particularly the reservoir function It was therefore considered of interest to further explore the role of the gastric extrinsic inhibitory nerve supply in the regulation of gastric motility, particularly during various types of receptive relaxation The interest was focused on the following questions:

1) Can vagal relaxation of the stomach be elicited from mechanoreceptors in the gastric wall? To answer this question the effect of distension either of the entire stomach (I) or selectively of the gastric antrum (II) was studied For the evaluation of the functional significance of such reflex effects particular attention was paid to the circumstances during which they were elicited

2) Can activation of gastric mechanoreceptors suppress gastric motility also through a non-vagal extrinsic reflex mechanism? This question was raised by an observation (in II) that antral distension could inhibit gastric contractions also after vagotomy The receptor mechanism and nervous pathway involved in this reflex phenomenon were then investigated (III)

3) Is the gastric receptive relaxation, which takes place during emesis mediated by any of the mentioned extrinsic inhibitory nerve supplies? To answer this question it was considered of interest to study gastric motility during emesis induced in two principally different ways, via nerves likely to act directly on the vomiting center (V) or by drug action on the medullary chemoreceptor trigger zone (IV) (see Introduction) Since the somatomotor vomiting response is suppressed by anesthetics (see Wang and Borison 1952) changes in gastric motility to emetic stimuli were studied also in unanesthetized decerebrate cats besides studies on anesthetized cats (IV V) as well as on conscious dogs (preliminarily reported in this context)

Parts of the present results have previously been preliminarily reported (Abrahamsson and Thorén 1970 Abrahamsson 1971 Abrahamsson Jansson and Martinson 1971 Abrahamsson 1973)

METHODOLOGICAL CONSIDERATIONS

One of the intentions of the present studies was to explore nervous mechanisms involved in the control of the gastric reservoir function It was therefore considered very important to use a method for recording of gastric motility which particularly well would reflect the volume changes of the gastric reservoir sections A recording technique, by which the volume of the stomach is measured at a fairly constant low transmural pressure could be expected to meet this requirement since marked volume changes occur only in the corpus-fundus part while antral volume changes ordinarily are relatively small (Code and Carlson 1968, own observations in II and III) Jansson and Martinson (1965) introduced such a volume recording method employed in the present studies, as modified by Jansson (1969 a) Gastric volume changes are recorded at a low transmural pressure by means of a large, thinwalled balloon introduced via the esophagus This method was suitable also for the present studies because the serious disturbances often induced by abdominal surgery can thus be avoided Such disturbances which at least in part are due to abnormal activation of suppressive reflexes otherwise will lead to a considerable loss of gastric tone (cf Jansson 1969 b and unpublished observations) which makes it difficult or impossible to reveal other types of suppressive influences on the motility

With the present volume recording technique, the term gastric tone is used to describe the current volume level upon which shortlasting phasic volume changes can be superimposed (cf Bozler 1948) The method used has the advantage that changes in the level of gastric reservoir tone can be followed over a very wide range This is in contrast to the conventional method of recording pressure in a balloon holding a fixed volume Usually this method only poorly reflects the true events (cf Martinson and Muren 1963 Jansson and Martinson 1965) since the necessary close contact between the balloon circumference and the stomach wall is easily interfered with thanks to the marked volume changes of the gastric corpus-fundus part In the present experiments both enhancements and suppressions (I-V) in gastric motility were readily revealed in full extent by the method of recording used It should here be noted however that the records reflect net changes in volume and that information of the precise type or site of e g local contractions cannot be obtained from such recordings alone; nor with the pressure recording method for that matter

In all experiments requiring intraabdominal preparation great care was taken to minimize the handling of the stomach and the intestine In studies II and III where reflex effects of antral distension was studied separation of the antrum from the gastric corpus and duodenum

was for this reason, made by cautiously placing ligatures outside the gastric wall Selective mechanical stimulation of the antrum was performed either by isotonic antral distension (fixed external pressure loads) or by isometric antral contractions i e the antrum was allowed to contract against a fairly constant volume Antral contractions were then induced either by close i a infusion of acetylcholine to the antrum (II) or by efferent electric stimulation of the vagal nerves (III) In the latter case contractions were of course also induced in other gastro-intestinal parts including corpus-fundus of the stomach which in fact was of advantage because the aim of these particular experiments was to study non-vagal reflex inhibitory effects on excitatory corpus-fundus motility

Most of the experiments were performed on cats anesthetized with chloralose In studies IV and V, where the gastric motility during vomiting was studied unanesthetized cats exposed to ischemic decerebration were also used which proved suitable for this purpose because the vomiting pattern can be easily induced in such animals

For details concerning stimulation of heart receptors (V) administration of apomorphine (IV) and other drugs (I-V) see the pertinent papers

RESULTS AND COMMENTS

1 Reflex activation of the vagal relaxatory fibres from gastric mechanoreceptors

From earlier studies on gastric motility it was clear that the vagal non-adrenergic relaxatory fibres to the stomach are involved in vago-vagal reflex mechanisms, induced either by electric afferent vagal stimulation (Jansson 1968 1969, Ohga et al 1969 1970) or by activation of esophageal mechanoreceptors by distension (Abrahamsson and Jansson 1969) The observation by Jansson (1969) that this type of vago-vagal gastric relaxation can be elicited by afferent vagal stimulation also at the diaphragm level suggested the existence of abdominal receptors that can reflexly engage the vagal relaxatory fibres

Reflex pathways In I it was shown that transient elevation of the reservoir connected to the gastric balloon caused gastric distension which was followed by a long-lasting phase of atropine-resistant gastric relaxation The nature of this relaxing phase closely mimicked that induced by reflex activation of the vagal relaxatory fibres by esophageal distension and it was abolished by vagal blockade Direct evidence that activation of gastric mechanoreceptors elicits vago-vagal gastric relaxation via the vagal relaxatory fibres was obtained in II where even slight antral distension elicited a prompt pronounced and long-lasting corpus-fundus relaxation To prove that the reflex vago-vagal gastric responses are due to activation of vagal non-adrenergic relaxatory fibres other known autonomic transmission mechanisms were blocked without interfering with the response which was eliminated first after vagal block No specific blocker for the non-adrenergic transmittor released by the relaxatory fibres is as yet known (Burnstock 1972) Thus the reflex vagal gastric relaxation elicited by distension of the gastric wall (I II) persisted after administration of the antiadrenergic drug ine and after atropine, blocking cholinergic fibre effects (cf Martinson 1965 Campbell 1966) as well as after cervical transection of the spinal cord It was however abolished by vagal blockade (cold blockade or nerve section) and was mimicked by the gastric reflex response to esophageal distension or direct efferent electric stimulation of the vagal relaxatory fibres

Further evidence that gastric receptor afferents mediate the vago-vagal gastric relaxation described was obtained by afferent electric stimulation of gastric nerve branches Such stimulation of nerves from various parts of the corpus (I II) as well as from the antrum (II) produced reflex gastric relaxation with similar characteristics as those described for the reflex response to gastric distension The results from I

and it thus suggest that the vagal relaxatory fibres can be reflexly activated by distension of virtually any part of the stomach In this connection it seems reasonable to assume that one factor of importance could be the distribution and density of involved receptors in the different parts of the stomach (see below)

Very little is as yet known about the central nervous pathways of the vago-vagal relaxatory reflex described here The vago-vagal reflex gastric relaxation, produced by afferent electric vagal stimulation persisted after spinal cord transection at the C2-level and after intercollicular transection (Jansson 1969 b) which shows that this vago-vagal reflex is relayed in the brain stem Nakazato and Ohga (1971) have confirmed that the reflex discharge in vagal efferents at the neck level elicited by afferent stimulation of the abdominal vagus is relayed in the brainstem in close vicinity to the obex

Receptor mechanism To understand the possible functional significance of the vago-vagal gastric relaxatory reflex in response to gastric distension described here one must consider what mechanical events may be capable to elicit the reflex gastric response

The receptor mechanism for this reflex was found to be very sensitive to distension of the gastric wall In animals exhibiting no initial vagal relaxatory influence on the stomach at very low distension pressures graded isotonic distension of the whole stomach at steady transmural pressure loads (I) induced such relaxatory vagal effects at distending pressures of 6–7 cm H_2O When the gastric antrum was distended selectively (II) vagal atropine-resistant corpus-fundus relaxation could be produced by distension pressures as low as 5 cm H_2O and at 10 cm H_2O the relaxatory response was always clearcut With stepwise increases of antral distending pressure up to 25–30 cm H_2O (II) correspondingly increased gastric relaxatory reflex responses could be produced and the reflex responses were quite reproducable In a few instances antral distending pressures higher than 30 cm H_2O were used and could then produce a slight further corpus-fundus inhibition in addition to that obtained at 30 cm H_2O However such high distension pressures were seldom used because they required a very tight ligature between antrum and corpus which for technical reasons was usually avoided

When the stomach holds food under natural circumstances it is subjected to distension by its contents over rather long periods It is therefore of interest to know whether the reflex vagal relaxatory response to gastric distension varies with the duration of the applied distension and to what an extent the receptor mechanisms adapt It was found (II) that a maintained reflex corpus-fundus relaxation was obtained for all durations of antral distension tried i e up to 10 min Thus the vagal receptor mechanism involved in this reflex is

RESULTS AND COMMENTS

1 Reflex activation of the vagal relaxatory fibres from gastric mechanoreceptors

From earlier studies on gastric motility it was clear that the vagal non-adrenergic relaxatory fibres to the stomach are involved in vago-vagal reflex mechanisms, induced either by electric afferent vagal stimulation (Jansson 1968 1969, Ohga et al 1969 1970) or by activation of esophageal mechanoreceptors by distension (Abrahamsson and Jansson 1969) The observation by Jansson (1969) that this type of vago-vagal gastric relaxation can be elicited by afferent vagal stimulation also at the diaphragm level suggested the existence of abdominal receptors that can reflexly engage the vagal relaxatory fibres

Reflex pathways In I it was shown that transient elevation of the reservoir connected to the gastric balloon caused gastric distension which was followed by a long-lasting phase of atropine-resistant gastric relaxation The nature of this relaxing phase closely mimicked that induced by reflex activation of the vagal relaxatory fibres by esophageal distension and it was abolished by vagal blockade Direct evidence that activation of gastric mechanoreceptors elicits vago-vagal gastric relaxation via the vagal relaxatory fibres was obtained in II where even slight antral distension elicited a prompt, pronounced and long-lasting corpus-fundus relaxation To prove that the reflex vago-vagal gastric responses are due to activation of vagal non-adrenergic relaxatory fibres other known autonomic transmission mechanisms were blocked without interfering with the response which was eliminated first after vagal block No specific blocker for the non-adrenergic transmittor released by the relaxatory fibres is as yet known (Burnstock 1972) Thus the reflex vagal gastric relaxation elicited by distension of the gastric wall (I II) persisted after administration of the antiadrenergic drug guanethidine and after atropine blocking cholinergic fibre effects (cf Martinson 1965 Campbell 1966) as well as after cervical transection of the spinal cord It was, however abolished by vagal blockade (cold blockade or nerve section) and was mimicked by the gastric reflex response to esophageal distension or direct efferent electric stimulation of the vagal relaxatory fibres

Further evidence that gastric receptor afferents mediate the vago-vagal gastric relaxation described was obtained by afferent electric stimulation of gastric nerve branches Such stimulation of nerves from various parts of the corpus (I II) as well as from the antrum (II) produced reflex gastric relaxation with similar characteristics as those described for the reflex response to gastric distension The results from I

and II thus suggest that the vagal relaxatory fibres can be reflexly activated by distension of virtually any part of the stomach In this connection it seems reasonable to assume that one factor of importance could be the distribution and density of involved receptors in the different parts of the stomach (see below)

Very little is as yet known about the central nervous pathways of the vago-vagal relaxatory reflex described here The vago-vagal reflex gastric relaxation produced by afferent electric vagal stimulation persisted after spinal cord transection at the C2-level and after intercollicular transection (Jansson 1969 b) which shows that this vago-vagal reflex is relayed in the brain stem Nakazato and Ohga (1971) have confirmed that the reflex discharge in vagal efferents at the neck level elicited by afferent stimulation of the abdominal vagus is relayed in the brainstem in close vicinity to the obex

Receptor mechanism To understand the possible functional significance of the vago-vagal gastric relaxatory reflex in response to gastric distension described here one must consider what mechanical events may be capable to elicit the reflex gastric response

The receptor mechanism for this reflex was found to be very sensitive to distension of the gastric wall In animals exhibiting no initial vagal relaxatory influence on the stomach at very low distension pressures graded isotonic distension of the whole stomach at steady transmural pressure loads (I) induced such relaxatory vagal effects at distending pressures of 6-7 cm H_2O When the gastric antrum was distended selectively (II) vagal atropine-resistant corpus-fundus relaxation could be produced by distension pressures as low as 5 cm H_2O and at 10 cm H_2O the relaxatory response was always clearcut With stepwise increases of antral distending pressure up to 25-30 cm H_2O (II) correspondingly increased gastric relaxatory reflex responses could be produced and the reflex responses were quite reproducable In a few instances antral distending pressures higher than 30 cm H_2O were used and could then produce a slight further corpus-fundus inhibition in addition to that obtained at 30 cm H_2O However such high distension pressures were seldom used because they required a very tight ligature between antrum and corpus which for technical reasons was usually avoided

When the stomach holds food under natural circumstances it is subjected to distension by its contents over rather long periods It is therefore of interest to know whether the reflex vagal relaxatory response to gastric distension varies with the duration of the applied distension and to what an extent the receptor mechanisms adapt It was found (II) that a maintained reflex corpus-fundus relaxation was obtained for all durations of antral distension tried i e up to 10 min Thus the vagal receptor mechanism involved in this reflex is

evidently of a slowly adapting character Except for one single vagal afferent unit of subserosal origin found in a cat by Iggo (1957 b), all slowly adapting gastric mechanoreceptors with vagal afferents described so far are so called tension receptors", being functionally in series with the gastric smooth muscle cells (see Leek 1971)

These data from electrophysiologic receptor studies make it likely that also the receptors responsible for the vago-vagal gastro-gastric relaxation would be tension receptors This means that if mere increases of antral wall tension are produced without concomitant changes in antral volume, this would also lead to a reflex vagal corpus-fundus relaxation In animals prepared to allow close i a acetylcholine (Ach) injections to the antrum region and still maintaining a relatively high corpus-fundus tone (II) such injections if made to the deflated antrum, had only slight reflex relaxatory effect on corpus-fundus However similar injections performed to the inflated antrum produced marked increases in antral wall tension and this was followed by a pronounced and long-lasting corpus-fundus relaxation (II Fig 5) that remained also after guanethidine Such injections of acetylcholine had no relaxatory effect on corpus-fundus after vagotomy even if corpus-fundus tone was high (unpublished observations)

There are some restrictions concerning the conclusions that can be drawn from the experiments with Ach injections if taken alone (see II) However when also the other results from mechanical antral stimulation are added, and integrated with the mentioned data and with the results of electrophysiologic receptor studies it seems reasonable to conclude that slowly adapting gastric tension receptors are responsible for elicitation of the vago-vagal gastro-gastric relaxation described here Rough handling of the stomach and the intestines leads, as mentioned to an often profound decrease of gastric tone (Jansson 1969 b) making it difficult to reveal further inhibition It is likely that even the cautious abdominal preparation employed in II may have induced some loss of gastric tone This means that the pronounced corpus-fundus relaxation in response to antral distension may, under completely ...matic conditions be still more pronounced than that obtained in the present experiments

2 Reflex activation of adrenergic splanchnic nerve fibres from gastric mechanoreceptors

In various situations gastric motility is suppressed by splanchnic nervous activity (see e g Thomas and Baldwin 1968) The earlier concept was that such suppression occurred in pathophysiological situations only However even slight distension of the small intestine induces reflex inhibition of gastric motility due to reflex activation of adrenergic fibres in the splanchnic nerves a reflex mechanism which in all

likelihood is of spinal nature (Jansson and Martinson 1966) It is on the other hand not assessed whether activation of mechanoreceptors in the gastric wall itself can also influence gastric motility via an extrinsic adrenergic reflex mechanism

In study II it was observed that already slight antral distension produced inhibition of the corpus-fundus contractions, also after vagotomy Since gastric distension had been found to produce reflex activation of the vagal non-adrenergic relaxatory fibres in a vago-vagal manner (I II) this observation suggested the existence of a dual gastro-gastric inhibitory reflex arrangement one being vagal the other non-vagal To gain information about the latter mechanism e g to elucidate the conditions under which it may be activated and to explore the neural pathways involved an analysis was made in a similar manner as described for the vagal gastro-gastric relaxation i e with particular regard to the reflex pathways and receptor mechanisms involved

Reflex pathways When rhythmic corpus-fundus contractions (spontaneous or elicited by vagal stimulation) were present in vagotomized preparations even slight isotonic antral distensions at pressures of 5-20 cm H_2O promptly inhibited or markedly reduced the prevailing contractions (III) This non-vagal gastro-gastric reflex inhibition was shown to be mediated by an adrenergic mechanism because it was completely abolished by guanethidine 3-5 mg/kg Such doses do not affect the vagally mediated relaxatory gastric response to gastric distension (I II) but block effectively reflex adrenergic inhibitions of gastric motility (Jansson and Martinson 1966)

It was a constant finding that moderate antral distensions never produced inhibition of corpus-fundus motility in vagotomized animals if corpus-fundus contractions were absent (Fig 2 3 4 in III) This was the case whether the prevailing corpus-fundus tone was high or low All these observations are in accord with those of Jansson and Martinson (1966) and Jansson (1969 b) which suggest that the splanchnic adrenergic fibres to the stomach affect motility mainly by inhibition of intramural cholinergic neurons in the gastric wall The observation that in the absence of corpus-fundus contractions intense antral distension caused only a slight and greatly delayed (30 sec or more) increase of corpus-fundus volume also fits well with such mode of action of adrenergic fibres to the stomach In the latter case the slight and delayed inhibition may have been caused by overflow of the adrenergic transmittor liberated at some distance from the smooth muscle cells As pointed out in Introduction there is now considerable evidence that the main locus of action for the adrenergic fibres in the gastro-intestinal tract is the intramural myenteric cholinergic neurons, instead of a direct action on the smooth muscle cells except concerning

the vascular innervation

A question of interest was at which level the inhibitory corpus-fundus response to antral distension was relayed Could it be mediated solely by pathways in the gastric wall via peripheral autonomic ganglia or did it require the participation of central nervous structures? The effect of spinal anesthesia in III clearly shows that this reflex response as elicited by antral distension or afferent electrical antral nerve stimulation, cannot be mediated solely by intramural gastric connections or by peripheral autonomic ganglia, but requires intact connections with the spinal cord It was however, present after cervical transection of the spinal cord suggesting a spinal organization of the reflex which of course, by no means excludes the possibility of supraspinal modulating influences This gastro-gastric adrenergic inhibitory reflex shows an arrangement similar to the intestino-intestinal inhibitory reflex (Johansson and Langston 1964) and the intestino-gastric inhibitory reflex (Jansson and Martinson 1966) since both these latter reflex mechanisms are also abolished by spinal anesthesia It has been shown that the intestino-intestinal inhibitory reflex can be markedly modulated by influences from supraspinal structures (Johansson Jonsson and Ljung 1965 1968) which is a likely possibility also for the gastro-gastric adrenergic inhibitory reflex Such supraspinal controls may be necessary for a proper coordination and sensitization of the two separate gastro-gastric inhibitory reflexes conveyed via vagal nerves in the bulb and via splanchnic fibres in the spinal cord respectively

Receptor mechanism. To understand the conditions under which the gastro-gastric adrenergic inhibitory reflex may influence gastric motility it was also considered of interest to explore which mechanical events in the gastric wall that may initiate such reflex activity Reports from electrophysiologic studies of gastric mechanoreceptors with splanchnic afferents are at present not available

From the experiments in which the effect of isotonic antral distension on corpus-fundus contractions were studied it appeared that the receptor mechanism involved was very sensitive to distension Thus antral distension pressures of only 5–6 cm H_2O were sometimes effective in eliciting reflex inhibitory responses and pronounced inhibition could regularly be obtained by distensions at 10 cm H_2O Further when the antral distension was increased in a stepwise fashion (Fig 4 in III) a well graded reflex inhibitory response was seen i e the degree of activation of the receptor mechanism and the reflex activation of the efferent adrenergic fibres must have been intimately related to the degree of distension

As pointed out in the description of the vagal gastro-gastric relaxatory reflex, it may be of great importance whether a gastro-gastric reflex affecting motility is elicited from a slowly or rapidly adapting

receptor mechanism The results in III clearly indicate that the gastro-gastric adrenergic inhibitory reflex is like the vagal gastro-gastric relaxatory reflex induced from slowly adapting mechanoreceptor mechanisms because the inhibitory response to antral distension were well maintained for all durations of antral distension employed (10 min) Also when antral contractions were occasionally absent for 2-3 min during a passive antral distension i e if the antral wall tension was kept constant during this period the reflex adrenergic inhibitory response was maintained throughout the distension strongly suggesting an initiation from slowly adapting mechanoreceptors Consequently it can be inferred that also the gastro-gastric adrenergic inhibitory reflex is activated and can affect the neural excitatory influence on gastric motility as long as the stomach is sufficiently distended by its contents

From the account given above it is evident that "passive gastric distension can elicit the gastro-gastric adrenergic inhibitory reflex Available data from electrophysiological studies on splanchnic nerve afferents did not give any suggestions whether the receptors involved were likely to be activated also by contractions of the gastric wall i e if they behave as tension receptors (cf Leek 1971) In study III isometric antral contractions were induced by switching from an isotonic to an isometric antral condition The antral pressure waves then appeared with a frequency of 4–5/min It was clearly shown that these isometric antral contractions produced a reflex non-vagal inhibition of prevailing corpus-fundus contractions There was a fairly constant temporal relationship (4–6 sec) between the antral pressure rise and the subsequent corpus-fundus inhibition When a series of antral contractions was induced the corpus-fundus volume increased with the first antral waves to reach a partially suppressed state which was then maintained as long as the antrum was allowed to contract isometrically Furthermore the inhibition produced in the corpus-fundus was for equal average antral distending volumes more pronounced with isometric than with isotonic antral distensions indicating that the distending volume could not be the only factor determining the degree of receptor activation for this reflex The gastro-gastric adrenergic inhibitory reflex is thus activated both by passive distension and by isometric contraction of the gastric wall i e it seems to be initiated mainly from so called tension receptors

Concerning the vagal gastro-gastric reflex mechanism (I, II) it was possible to relate this reflex and its mode of activation to fairly well known gastric mechanoreceptors with afferents in the vagal nerve In contrast there are no similar investigations described in the literature concerning gastric mechanoreceptors with afferents in the splanchnic nerves which can be applied to the present analysis of the gastro-gastric adrenergic reflex mechanism The results presented in III suggest

a tension receotor mechanism being involved possibly of a similar type as that described for vagal gastro-intestinal receptors (Iggo 1955 1957 a Leek 1969, Mei 1970 Harding and Leek 1972 a) Similar splanchnic nerve afferents with receptors embedded within the smooth muscle layer of the viscus have as yet not been described On the other hand, gastric mechanoreceptors located just beneath the serosa and with splanchnic nerve afferents, have recently been found by Morrison (personal communication) and in the antral region they give a slowly adapting discharge in response to distension Also antral contractions could activate these receptors which according to Morrison, behave as tension receptors, despite their superficial location in the gastric wall Thus, these subserosal receptors may be involved in this gastro-gastric inhibitory reflex although other types of receptors, e g mechanoreceptors placed functionally in series with the smooth muscle cells cannot at present be excluded

3 Role of the gastro-gastric suppressive reflexes in regulation of gastric motility

General considerations The results in I II and III showed the existence of two separate gastro-gastric reflexes with suppressive effects on gastric motility The arrangement of these two extrinsic gastric reflex mechanisms is schematically illustrated in Fig 1

The similarities between the receptor mechanisms for the two reflexes - the low threshold the slowly adapting character and the tension receptor characteristics - suggest that the two reflexes under physiological circumstances are often activated in close parallel as was the case in connection with antral distension (II III) An interesting aspect is the richness of mechanoreceptors in the antral region (Paintal 1954 Iggo 1955 1957 a Sharma et al 1972) which suggests that this is an important region for extrinsic nervous control mechanisms It has been shown (Iggo 1955 Leek 1969) that slowly adapting tension receptors with vagal afferents exhibit a greater afferent discharge for a given wall tension when this is developed by isometric contraction than by mere passive distension The effect of isometric antral distension on corpus-fundus motility dealt with in III (Fig 7) suggests that antral contractions are very effective in elicitation of the gastro-gastric adrenergic inhibitory reflex The antrum is the gastric region where the most well-defined isometric contractions are likely to occur especially during digestion of solid food There are thus several reasons to believe that antral mechanoreceptors of the tension type are particularly important in the reflex nervous control of gastric motility There is, on the other hand no reason to believe that antral mechanoreceptors are the only ones of importance for gastro-gastric inhibitory reflexes Slowly adapting tension receptors have been found also in the

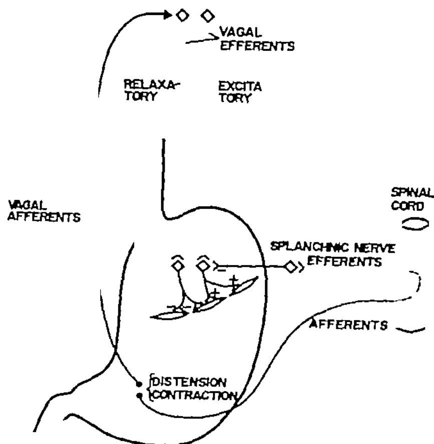


Fig 1 Innervation of the gastric smooth muscles Arrangement of the two gastro-gastric suppressive reflex mechanisms in the vagal and splanchnic nerves respectively For further details see text

proximal parts of the stomach (Paintal 1954 Iggo 1955 1957 a Mei 1970) and electric afferent stimulation of corpus nerves (I II) elicits vago-vagal reflex gastric relaxation in the same manner as stimulation of antral nerves

From a functional point of view the most marked difference between the two suppressive gastro-gastric reflexes seems to be on the efferent side (Fig 1) As shown in III the gastro-gastric adrenergic inhibitory reflex acts by inhibiting gastric contractions likely by inhibition of intramural cholinergic excitatory activity This means that the adrenergic gastro-gastric reflex can inhibit gastric motility only

a tension receptor mechanism being involved possibly of a similar type as that described for vagal gastro-intestinal receptors (Iggo 1955, 1957 a Leek 1969 Mei 1970 Harding and Leek 1972 a) Similar splanchnic nerve afferents with receptors embedded within the smooth muscle layer of the viscus have as yet not been described On the other hand gastric mechanoreceptors located just beneath the serosa and with splanchnic nerve afferents have recently been found by Morrison (personal communication) and in the antral region they give a slowly adapting discharge in response to distension Also antral contractions could activate these receptors which according to Morrison behave as tension receptors despite their superficial location in the gastric wall Thus these subserosal receptors may be involved in this gastro-gastric inhibitory reflex although other types of receptors e g mechanoreceptors placed functionally in series with the smooth muscle cells cannot at present be excluded

3 Role of the gastro-gastric suppressive reflexes in regulation of gastric motility

General considerations The results in I II and III showed the existence of two separate gastro-gastric reflexes with suppressive effects on gastric motility The arrangement of these two extrinsic gastric reflex mechanisms is schematically illustrated in Fig 1

The similarities between the receptor mechanisms for the two reflexes - the low threshold the slowly adapting character and the tension receptor characteristics - suggest that the two reflexes under physiological circumstances are often activated in close parallel as was the case in connection with antral distension (II III) An interesting aspect is the richness of mechanoreceptors in the antral region (Paintal 1954 Iggo 1955 1957 a Sharma et al 1972) which suggests that this is an important region for extrinsic nervous control mechanisms It has been shown (Iggo 1955 Leek 1969) that slowly adapting tension receptors with vagal afferents exhibit a greater afferent discharge for a given wall tension when this is developed by isometric contraction than by mere passive distension The effect of isometric antral distension on corpus-fundus motility dealt with in III (Fig 7), suggests that antral contractions are very effective in elicitation of the gastro-gastric adrenergic inhibitory reflex The antrum is the gastric region where the most well-defined isometric contractions are likely to occur especially during digestion of solid food There are thus several reasons to believe that antral mechanoreceptors of the tension type are particularly important in the reflex nervous control of gastric motility There is on the other hand no reason to believe that antral mechanoreceptors are the only ones of importance for gastro-gastric inhibitory reflexes Slowly adapting tension receptors have been found also in the

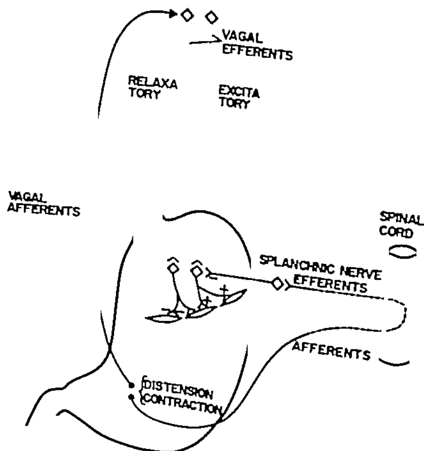


Fig 1 Innervation of the gastric smooth muscles Arrangement of the two gastro-gastric suppressive reflex mechanisms in the vagal and splanchnic nerves respectively For further details see text

proximal parts of the stomach (Paintal 1954 Iggo 1955 1957 a [illegible] 1970) and electric afferent stimulation of corpus nerves (I II) el[illegible] vago-vagal reflex gastric relaxation in the same manner as stimulation of antral nerves

From a functional point of view the most marked difference between the two suppressive gastro-gastric reflexes seems to be on the efferent side (Fig 1) As shown in III the gastro-gastric adrenergic inhibitory reflex acts by inhibiting gastric contractions likely [illegible] inhibition of intramural cholinergic excitatory activity This means [illegible] the adrenergic gastro-gastric reflex can inhibit gastric motility [illegible]

to the extent that the motility is dependent on activity of intramural cholinergic neurons, since the adrenergic fibres appear to have no significant direct influence on the gastric smooth muscles The vagal gastro-gastric relaxatory reflex, on the other hand utilizing the vagal non-adrenergic relaxatory fibres as the efferent link is not blocked by atropine and can therefore greatly affect the gastric smooth muscle cells whenever they are active whether this activity is due to intramural cholinergic nervous activity or is myogenic in origin (I II cf Jansson and Martinson 1965) From this account it is obvious that the suppressive effects of each of two gastro-gastric reflexes may quantitatively differ greatly (see e g Fig 1 in III)

It is at present not possible to definitely say whether there are also any qualitative differences in the responses of the two gastro-gastric reflexes, apart from such as are natural from their different modes of action Direct inspection of the stomach during antral distension (III) revealed that the gastro-gastric adrenergic inhibitory reflex can produce both a considerable widening of the gastric reservoir part and an inhibition of peristaltic corpus waves as long as a cholinergic nervous activity is present It is clear that also the vagal gastro-gastric reflex can produce a general and really profound widening of corpus-fundus as judged e g from the recordings in atropinized animals (I II) It is, on the other hand at present more uncertain what effect activation of the vagal relaxatory fibres may have on gastric peristaltic activity However studies by Jansson and Martinson (1965) show that the vagal cholinergic fibres can still induce considerable contractions superimposed upon the marked widening that a relaxatory fibre activation induces Further in studies of the effect of long-lasting esophageal distensions (unpublished observation) the gastric volume could be profoundly increased for 8-10 min during which time marked rhythmic gastric contractions were still present This may suggest a differentiation between effects on the general gastric tone and effects [illegible] staltic activity The crucial problem in this connection is cholinergic agents must be given to prove that an effect [illegible]lated by the vagal non-adrenergic relaxatory fibres and [illegible]urb the normal gastric peristalsis A blocking drug [illegible] effect on the vagal relaxatory fibres could help to s[illegible] present problems

From the above description it is obvious that [illegible]cumstances with intact reflexes activation of [illegible] noreceptors by distension and contraction a reflex [illegible]duced that affects both inherent gastric tone and phasic contractions that depend on cholinergic inf[illegible] ever of great importance to know the qualitative effects of the respective reflexes to understand the [illegible]ference with any of these control mechanisms may

effect vagotomy or administration of antiadrenergic drugs may have on gastric reservoir and emptying functions

Control of gastric reservoir function Very little has been known how the ability of the stomach to receive large volumes without substantial pressure rise is controlled It is an old observation (see Thomas and Baldwin 1968) that when the stomach starts to digest a meal there is a vagally increased gastric contractile activity, essential for an adequate emptying function One may then ask how the stomach can be able to still keep its contents at a fairly low reservoir pressure This apparently calls for adjustment mechanisms capable to balance the vagal excitatory drive on the stomach The experimental findings of Martinson Jansson and coworkers in cats referred to earlier have here furnished data that provide a background to a reasonable explanation Further recent studies in man on the effect of vagotomy (Aune 1969 Köster and Madsen 1970 Stadaas and Aune 1970) clearly suggest that participation of the vagal nerves is necessary for this receptive mechanism

Experiments in I showed that there was no or only little evidence of a vagal relaxatory effect on the stomach at very low degrees of filling (Fig 3 in I) but at even moderate degrees of increased filling the vagal non-adrenergic relaxatory fibres became activated and kept the stomach in a relaxed state It was found that the participation of the vagal relaxatory supply could at a given intragastric pressure increase the capacity of the stomach to hold volumes by 50-90% In experiments with transient gastric distensions (Fig 2 in II) it was shown that the capacity of the stomach to receive volumes is markedly increased as a result of a reflex activation of the vagal relaxatory fibres which is induced by the filling per se It was also observed that blockade of this vagal reflex mechanism (vagotomy or cold blockade) led to a decreased gastric inflow when the stomach was in connection with a water reservoir (Fig 2 in II) i e gastric tone increased after vagal blockade, if tone is here defined as resistance to inflow to the viscus according to Lipkin et al (1962) The results in I thus indicate the existence of a vago-vagal gastro-gastric relaxatory reflex being functionally a storage reflex (see below)

The receptor mechanism for this reflex as analysed in II showed properties particularly suitable for a gastro-gastric storage reflex Thus it showed no tendency to adapt suggesting that it can maintain a reflex inhibition of the gastric smooth muscles as long as the stomach is filled Furthermore the tension receptor property makes the reflex able to protect the reservoir from excessive pressure rises whether induced by passive filling or by wall contractions The existence of such a vagal gastro-gastric relaxatory reflex explains why vagotomy leads to an increased gastric tone as observed in pressure

to the extent that the motility is dependent on activity of intramural cholinergic neurons since the adrenergic fibres appear to have no significant direct influence on the gastric smooth muscles The vagal gastro-gastric relaxatory reflex, on the other hand utilizing the vagal non-adrenergic relaxatory fibres as the efferent link is not blocked by atropine and can therefore greatly affect the gastric smooth muscle cells whenever they are active whether this activity is due to intramural cholinergic nervous activity or is myogenic in origin (I II cf Jansson and Martinson 1965) From this account it is obvious that the suppressive effects of each of two gastro-gastric reflexes may quantitatively differ greatly (see e g Fig 1 in III)

It is at present not possible to definitely say whether there are also any qualitative differences in the responses of the two gastro-gastric reflexes apart from such as are natural from their different modes of action Direct inspection of the stomach during antral distension (III) revealed that the gastro-gastric adrenergic inhibitory reflex can produce both a considerable widening of the gastric reservoir part and an inhibition of peristaltic corpus waves as long as a cholinergic nervous activity is present It is clear that also the vagal gastro-gastric reflex can produce a general and really profound widening of corpus-fundus as judged e g from the recordings in atropinized animals (I II) It is on the other hand at present more uncertain what effect activation of the vagal relaxatory fibres may have on gastric peristaltic activity However studies by Jansson and Martinson (1965) show that the vagal cholinergic fibres can still induce considerable contractions superimposed upon the marked widening that a relaxatory fibre activation induces Further in studies of the effect of long-lasting esophageal distensions (unpublished observation) the gastric volume could be profoundly increased for 8-10 min during which time marked rhythmic gastric contractions were still present This may suggest a differentiation between effects on the general gastric tone and effects on peristaltic activity The crucial problem in this connection is that anticholinergic agents must be given to prove that an effect really is me-... by the vagal non-adrenergic relaxatory fibres and such drugs disturb the normal gastric peristalsis A blocking drug with selective effect on the vagal relaxatory fibres could help to solve many of the present problems

From the above description it is obvious that under ordinary circumstances with intact reflexes activation of gastric and antral mechanoreceptors by distension and contraction a reflex suppression is induced that affects both inherent gastric tone and the superimposed phasic contractions that depend on cholinergic influences It is however of great importance to know the qualitative and quantitative effects of the respective reflexes to understand the effect that an interference with any of these control mechanisms may have e g what

to the bladder being inhibited in the spinal cord (Groat and Ryall 1969) while the gastric intramural excitatory neurons are suppressed by the gastro-gastric adrenergic inhibitory reflex (III) There are however also large differences in the control mechanisms of the two organs which is not surprising considering their differences in function with respect to both filling and emptying

Possible role of the gastro-gastric inhibitory reflexes in regulation of gastric mixing and emptying In the present experiments some observations were made that may be relevant for the gastric mixing and emptying processes Both the vagal and the adrenergic gastro-gastric reflexes proved to be intensely activated when high tension was developed in the antral wall which occurs when the antrum contracts during gastric digestion of solid food (Thomas 1957) During such digestion the suppressive reflex effects on the reservoir may be especially prominent which allows for more contents to be retropelled to the corpus during antral contractions less being emptied into the duodenum (see Introduction and Code and Carlson 1968) A consequence of the described reflex responses may therefore be prolonged retention of gastric contents with better mixing and gastric digestion The pyloric resistance to passage (Nelsen and Kohatsu 1971) in combination with the abundance of antral mechanoreceptors (Paintal 1954 Iggo 1955 1957 a Sharma et al 1972) that effectively suppress reflexly the reservoir (II III) allows the prepyloric region to act almost like a baroreceptor station initiating a gastro-gastric feed-back regulation simultaneously with its pumping function

Experimental studies that can be applied to this hypothesis seem to be sparse Results from truncal or selective gastric vagotomy are not relevant because these procedures affect both the reservoir function and the pump regulation However recent studies (Moberg et al 1972) concerning a selective vagal denervation of the gastric reservoir (selective proximal vagotomy or parietal cell vagotomy) suggest that the consequently impaired reservoir function (Martinson and Jahnberg personal communication) may result in an increased gastric emptying rate There are also reports of accelerated gastric emptying after splanchnic nerve section (Barron and Curtis 1937 Alvarez 1948 p 278) suggesting that splanchnic nerve fibres may under ordinary circumstances suppress gastric emptying

From what has been stated above it follows that one important function of the reflex gastro-gastric receptive relaxation may be to maintain the gastric emptying rate at a suitable level

volume investigations (Aune 1969 Stadaas and Aune 1970, Köster and Madsen 1970, Carter et al 1972), and why symptoms of impaired gastric reservoir function such as early satiety and epigastric fullness may ensue after vagotomy (Aune 1969)

The old concept that vagotomy leads to atony of the stomach (see e g Alvarez 1948) has apparently not been based on measurements where the pressure-volume relationships were considered but rather on the fact that gastric emptying is also impaired which may itself lead to retention These disturbances may thus depend on an impaired antral pumping (Nelsen et al 1967) rather than on changes in overall gastric tone which, if anything would tend to accelerate gastric emptying

The gastro-gastric adrenergic inhibitory reflex (III) also exhibits marked suppressive effects on gastric reservoir function and shows receptor properties similar to the vagal gastro-gastric reflex The adrenergic inhibitory reflex elicited by mechanical events partly generated by vagal activity (e g Figs 6 and 7 in III) has an arrangement well suited for damping the vagal excitatory effect during gastric digestion Therefore the gastro-gastric adrenergic reflex may normally have a substantial suppressive effect on the gastric reservoir

It is evident that the increased gastric tone after vagotomy cannot be due to direct interference with the adrenergic reflex mechanism Instead this reflex mechanism may after vagotomy contribute to the gastric reservoir capacity by modulating the activity of the intramural cholinergic neurons but it cannot alone induce enough of gastric relaxation to maintain a normal reservoir capacity

In the process of gastric filling during food intake the stomach becomes relaxed already when food passes the pharyngeo-esophageal region (Cannon and Lieb 1911, Lind et al 1961) due to reflex activation of vagal relaxatory fibres (Abrahamsson and Jansson 1969) i e even before the food has reached the stomach However once the food intake is finished the gastric relaxatory effect of this type of reflex fades off In this situation the gastro-gastric suppressive reflexes (I, II III) take over continuously modulating gastric volume so that the stomach is kept in a suitably relaxed state The vagal non-adrenergic relaxatory fibres are important for both these types of gastric receptive relaxation

There are some interesting similarities between this reflex nervous control of the reservoir function of the stomach and that of the urinary bladder During filling the urinary bladder is influenced by an inhibitory spinal storage reflex (Edvardsen 1967 Groat and Lalley 1972) constituted by pelvic nerve afferents, activated from intramural tension receptors and with adrenergic fibres in the hypogastric nerves as the efferent link Further in both reservoir organs the effect of the excitatory fibres are suppressively controlled the excitatory pelvic neurons

to the bladder being inhibited in the spinal cord (Groat and Ryall 1969) while the gastric intramural excitatory neurons are suppressed by the gastro-gastric adrenergic inhibitory reflex (III) There are however also large differences in the control mechanisms of the two organs which is not surprising considering their differences in function with respect to both filling and emptying

Possible role of the gastro-gastric inhibitory reflexes in regulation of gastric mixing and emptying In the present experiments some observations were made that may be relevant for the gastric mixing and emptying processes Both the vagal and the adrenergic gastro-gastric reflexes proved to be intensely activated when high tension was developed in the antral wall, which occurs when the antrum contracts during gastric digestion of solid food (Thomas 1957) During such digestion the suppressive reflex effects on the reservoir may be especially prominent which allows for more contents to be retropelled to the corpus during antral contractions less being emptied into the duodenum (see Introduction and Code and Carlson 1968) A consequence of the described reflex responses may therefore be prolonged retention of gastric contents with better mixing and gastric digestion The pyloric resistance to passage (Nelsen and Kohatsu 1971) in combination with the abundance of antral mechanoreceptors (Paintal 1954 Iggo 1955 1957 a Sharma et al 1972) that effectively suppress reflexly the reservoir (II III) allows the prepyloric region to act almost like a baroreceptor station initiating a gastro-gastric feed-back regulation simultaneously with its pumping function

Experimental studies that can be applied to this hypothesis seem to be sparse Results from truncal or selective gastric vagotomy are not relevant because these procedures affect both the reservoir function and the pump regulation However recent studies (Moberg et al 1972) concerning a selective vagal denervation of the gastric reservoir (selective proximal vagotomy or parietal cell vagotomy) suggest that the consequently impaired reservoir function (Martinson and Jahnberg personal communication) may result in an increased gastric emptying rate There are also reports of accelerated gastric emptying after splanchnic nerve section (Barron and Curtis 1937 Alvarez 1948 p 278) suggesting that splanchnic nerve fibres may under ordinary circumstances suppress gastric emptying

From what has been stated above it follows that one important function of the reflex gastro-gastric receptive relaxation may be to maintain the gastric emptying rate at a suitable level

4 Control of gastric receptive relaxation in emesis

Preceding vomiting a profound receptive relaxation of the upper part of the stomach occurs, in association with an orad transport of gastrointestinal contents to this gastric reservoir region It was considered of interest to see whether this type of gastric receptive relaxation is controlled by similar mechanisms as that induced from e g pharyngo-esophageal structures (Abrahamsson and Jansson 1969) or from gastric mechanoreceptors (I II III) Since anesthetics interfere greatly with the emetic somatomotor response (see Wang and Borison 1952), gastric motility during vomiting was studied also in unanesthetized decerebrate cats (IV V) and in conscious dogs Vomiting was elicited either by the centrally acting emetic drug apomorphine (IV and in dog experiments) or by activation of vagal cardiac afferents (V) likely to be activated in some pathophysiological situations where nausea is common (Abrahamsson and Thorén 1972 Öberg and Thorén 1972 Thoren 1972)

In the unanesthetized decerebrate cats administration of apomorphine (IV) or electric afferent stimulation of the right cardiac nerve (V) produced a pronounced gastric relaxation followed by forceful somatomotor vomiting movements which rapidly emptied the gastric balloon When the retching or vomiting movements stopped the stomach volume returned to the relaxed state which in both types of experiments only slowly faded off Especially when apomorphine had been given the regain of gastric tone was often very slow sometimes requiring 1-2 hours for full recovery to control volume For both types of emesis-induction in unanesthetized cats a pronounced gastric relaxation could occur also without any subsequent retching response This was the rule when relatively small doses of apomorphine was given (IV) or when the cardiac nerve was stimulated at frequencies below 10 Hz (V)

Gastric motility during apomorphine-induced emesis in the awake state was also studied in two well-trained dogs with chronic gastric fistulae and in which the gastric volume recording method could be employed Fig 2 shows how a dose of apomorphine (10 µg/kg s c) just above the emetic threshold dose for dogs (Niemegeers 1971), produces a response very similar to that obtained in cats (in IV Fig 1 B) When vomiting occurred it was always preceded by a pronounced gastric relaxation and the subsequent somatomotor vomiting movements rapidly emptied the gastric balloon Gastric relaxation was not always associated with vomiting movements (Fig 2 left part) but also during this phase of gastric relaxation the dog postured with lowered head in the same characteristic manner as when complete vomiting actually appeared These observations in cat and dog experiments are fully in accord with previous reports mainly X-ray studies, that a pronounced gastric relaxation occurs early in the vomiting act (Openchowski 1889

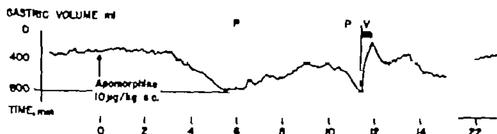


Fig 2 Gastric relaxation and vomiting induced by apomorphine (10 µg s c) in an awake dog Gastric relaxation is observed 3 min after drug injection and 2 min later the dog postures (P) in the way characteristic for vomiting 10 min after drug injection a second phase of gastric relaxation appears during which vomiting (V) occurs leading to prompt decrease of gastric volume Gastric volume is recovered 22-23 min after drug injection

Cannon 1898 Hesse 1913) so that the proximal part of the stomach is ready to receive contents arriving from more distal gastrointestinal regions

Analyses in cats of this gastric relaxation in response to emetic stimuli showed that it was mediated by the vagal non-adrenergic relaxatory fibres both when elicited by apomorphine (IV) and by afferent stimulation of the vagal cardiac nerve (V) In both types of experiments the gastric response was obtained also after administration of anticholinergic and antiadrenergic drugs but was abolished by vagal section when performed at such levels as to interfere only with efferent nervous pathways to the stomach in the two types of experiments.

It appeared that splanchnic nerve mechanisms did not contribute much to the gastric volume increase obtained by apomorphine or cardiac nerve stimulation The absence of clearcut effects of these stimuli on vagally induced gastric contractions (IV V) indicates that splanchnic adrenergic fibres to the stomach are of minor importance for the volume response (cf Jansson and Martinson 1966, III) It should be noted however that the recording method used reflects overall gastric volume changes Thus there could within the stomach occur regional changes in motility that are not readily revealed by the present method e g distal parts of the stomach may show complex contractile patterns (Smith and Brizzee 1961 Lumsden and Holden 1969) which will not produce appreciable changes in overall gastric volume There is how-

ever little doubt that *during emesis a marked increase in gastric volume* is mediated by the vagal relaxatory fibres

The results from cats and dogs showed that marked gastric relaxation can be induced by apomorhine doses that are too small to induce vomiting Likewise clearcut gastric relaxation was produced by afferent cardiac nerve stimulation at frequencies well below those needed to produce vomiting movements When such apomorphine-induced gastric relaxation without definite vomiting occurred in the conscious dog the animal postured in the characteristic manner as when the gastric relaxation was accompanied by vomiting It is therefore likely that this gastric relaxation is a phenomenon associated with nausea This is supported by X-ray observations in man (Barclay 1936 Lumsden and Holden 1969) since a drop of the greater gastric curvature can be observed in subjects experiencing nausea

Since nausea is an experience dependent on cortical nervous activity there may be situations in which the medullary emetic mechanism is somewhat activated and mediating gastric relaxation without the subject experiencing nausea Wolf (1943) observed that gastric relaxation appeared several minutes before the subject became nauseated Ramsbottom and Hunt (1970) found that such small doses of apomorphine that do not induce nausea had a delaying effect on gastric *emptying It is interesting that this suppressive effect of apomorhine* on gastric emptying was eliminated by the antiemetic drug metoclopramide while this drug had no significant effect on gastric emptying when apomorphine had not been given

Upon afferent stimulation of the cardiac nerve (V) the circulatory depressor response was maximal already at a stimulation frequency of about 6 Hz while the gastric relaxatory response was maximal first at a frequency above 20 Hz This suggests that the receptor afferents involved essentially emanating from the left ventricle (see Abrahamsson and Thorén 1972) are primarily involved in cardiovascular regulation These receptors are however activated in situations where nausea

e g coronary artery occlusion (Thorén 1972) and the vasovagal syncope reaction (Öberg and Thorén 1972) It was also observed (V) that during coronary artery occlusion gastric relaxation was produced by reflex activation of the vagal relaxatory fibres Such relaxation was also produced by intrapericardial instillation of nicotine a *drug known to activate strongly the mentioned type of receptors Rather* strong activation of these heart receptors likely to produce nausea, was required to produce appreciable gastric reflex effects Thus these receptors are not likely to be involved in the normal regulation of gastric motility and consequently the gastric reflex response obtained from them can hardly represent a strictly physiological reflex regulatory mechanism but rather a pathophysiological variant

To summarize: the results described indicate that, irrespective of the way vomiting is produced – by action on the medullary chemoreceptor trigger zone (IV) or by direct activation of the vomiting center via autonomic afferents (V) – an emetic type of gastric receptive relaxation is in each instance induced by activation of the vagal relaxatory fibres to the stomach

5 The vagal non-adrenergic relaxatory fibres as mediators of gastric receptive relaxation

"Gastric receptive relaxation refers to the mode in which the stomach behaves during filling and is, as mentioned characterized by a pronounced capacity to receive large volumes with only slight increase in pressure Besides during deglutition gastric receptive relaxation is initiated both in response to direct intragastric filling and in the vomiting act as a corpus-fundus relaxation in this situation preceding the orad transport of gastro-intestinal contents

Evidence for the importance of the vagal non-adrenergic relaxatory fibres in the control of the mentioned types of gastric receptive relaxation has now accumulated (Abrahamsson and Jansson 1969 I II IV V) The modes in which this vagally mediated adaptation phenomenon is initiated are schematically illustrated in Fig 3 (p 30)

During ordinary gastric filling these vagal fibres are activated from the pharynx esophagus and the stomach itself and in the latter case the receptor mechanism is of the very slowly adapting type influenced both by gastric distension and contraction and thereby capable to keep the stomach in a relaxed state as long as it is filled (I II)

Swallowing and slight mechanical stimulation in the pharynx and esophagus (Abrahamsson and Jansson 1969) cause reflex activation of the vagal relaxatory fibres where the receptors are very sensitive to distension and slowly adapting (Fig 2 in Abrahamsson and Jansson 1969) responding moreover also to esophageal contractions (Mei 1970) Evidently also the esophageal mechanoreceptors seem to be tension receptors like the gastric ones mediating similar vago-vagal gastric relaxation

During emesis there is also a gastric receptive relaxation mediated by the vagal relaxatory fibres (IV V) and elicited also from structures others than the gastrointestinal tract such as vagal heart afferents (V) and by centrally acting emetic agents (IV) Thus also during the emetic act with widespread effects in many organs, the vagal relaxatory fibres mediate purposeful effects on the stomach

ever, little doubt that during emesis a marked increase in gastric volume is mediated by the vagal relaxatory fibres

The results from cats and dogs showed that marked gastric relaxation can be induced by apomorphine doses that are too small to induce vomiting Likewise, clearcut gastric relaxation was produced by afferent cardiac nerve stimulation at frequencies well below those needed to produce vomiting movements When such apomorphine-induced gastric relaxation without definite vomiting occurred in the conscious dog the animal postured in the characteristic manner as when the gastric relaxation was accompanied by vomiting It is therefore likely that this gastric relaxation is a phenomenon associated with nausea This is supported by X-ray observations in man (Barclay 1936 Lumsden and Holden 1969) since a drop of the greater gastric curvature can be observed in subjects experiencing nausea

Since nausea is an experience dependent on cortical nervous activity there may be situations in which the medullary emetic mechanism is somewhat activated and mediating gastric relaxation without the subject experiencing nausea Wolf (1943) observed that gastric relaxation appeared several minutes before the subject became nauseated Ramsbottom and Hunt (1970) found that such small doses of apomorphine that do not induce nausea had a delaying effect on gastric emptying It is interesting that this suppressive effect of apomorhine on gastric emptying was eliminated by the antiemetic drug metoclopramide while this drug had no significant effect on gastric emptying when apomorphine had not been given

Upon afferent stimulation of the cardiac nerve (V) the circulatory depressor response was maximal already at a stimulation frequency of about 6 Hz while the gastric relaxatory response was maximal first at a frequency above 20 Hz This suggests that the receptor afferents involved essentially emanating from the left ventricle (see Abrahamsson and Thorén 1972) are primarily involved in cardiovascular regulation These receptors are however activated in situations where nausea

e g coronary artery occlusion (Thorén 1972) and the vasovagal syncope reaction (Öberg and Thorén 1972) It was also observed (V) that during coronary artery occlusion gastric relaxation was produced by reflex activation of the vagal relaxatory fibres Such relaxation was also produced by intrapericardial instillation of nicotine a drug known to activate strongly the mentioned type of receptors Rather strong activation of these heart receptors likely to produce nausea, was required to produce appreciable gastric reflex effects Thus these receptors are not likely to be involved in the normal regulation of gastric motility and consequently the gastric reflex response obtained from them can hardly represent a strictly physiological reflex regulatory mechanism but rather a pathophysiological variant

SUMMARY

The extrinsic nervous control of gastric motility was studied mainly in cats with special reference to the role of the two suppressing nerve supplies the vagal non-adrenergic relaxatory fibres and the splanchnic adrenergic inhibitory fibres The interest was focused on the importance of these nerves in the control of gastric reservoir function (gastric receptive relaxation) The results can be summarized as follows:

1) A vago-vagal gastro-gastric relaxatory reflex was demonstrated the efferent link being constituted by the vagal non-adrenergic relaxatory fibres This reflex is activated already by slight distension of either the stomach as a whole or by selective distension of the gastric antrum The reflex response is a profound relaxation of the corpus-fundus reservoir part The receptor mechanism showed little tendency to adapt and behaved largely as tension receptors, thus responding both to gastric distension and contraction

2) The existence of a gastro-gastric adrenergic inhibitory reflex was demonstrated, with both afferent and efferent links conveyed by the splanchnic nerves and with the reflex coupling at the spinal level Also here the receptors particularly studied in the antral region behaved as slowly adapting tension receptors The efferent adrenergic link of the reflex acts essentially by inhibiting the excitatory cholinergic neurons in the gastric wall

3) These two distinctly separate suppressive gastro-gastric reflexes seem to be activated in close parallel in many situations and are jointly responsible for adjusting the gastric reservoir to its contents The vago-vagal gastro-gastric relaxatory reflex is here particularly important because of its pronounced relaxing effect that acts directly on the smooth muscle cells The considerable impairment of gastric reservoir capacity after vagotomy observed also in man supports this conclusion The gastro-gastric adrenergic inhibitory reflex complements however in an important way the vago-vagal reflex relaxation by modulating the activity of the cholinergic intramural excitatory neurons which during digestion elicit more or less rhythmic gastric contractions and propulsive movements Thus the two reflex mechanisms are likely to influence also gastric emptying by their effects on the aboral gastric pressure gradient

4) A pronounced gastric relaxation precedes vomiting in cats induced either by the centrally acting emetic drug apomorphine or by stimulation of heart receptor afferents In conscious dogs a similar gastric relaxation was observed in association with apomorphine induced nausea and vomiting This gastric relaxation was also mediated by the vagal

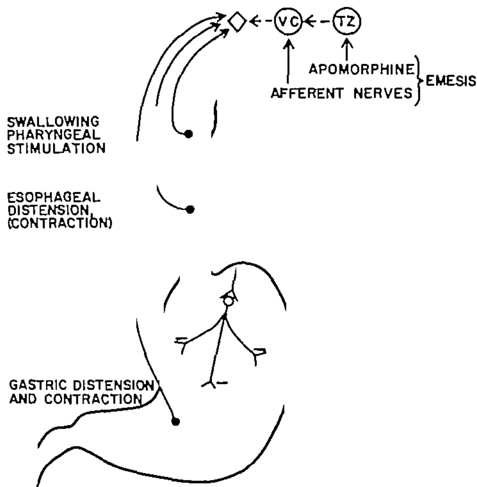


Fig 3 Schematic representation of reflex mechanisms producing gastric receptive relaxation *via* the vagal non-adrenergic relaxatory fibres to the stomach VC: vomiting centre TZ. trigger zone for centrally acting emetic agents

SUMMARY

The extrinsic nervous control of gastric motility was studied mainly in cats with special reference to the role of the two suppressing nerve supplies the vagal non-adrenergic relaxatory fibres and the splanchnic adrenergic inhibitory fibres The interest was focused on the importance of these nerves in the control of gastric reservoir function (gastric receptive relaxation) The results can be summarized as follows

1) A vago-vagal gastro-gastric relaxatory reflex was demonstrated the efferent link being constituted by the vagal non-adrenergic relaxatory fibres This reflex is activated already by slight distension of either the stomach as a whole or by selective distension of the gastric antrum The reflex response is a profound relaxation of the corpus-fundus reservoir part The receptor mechanism showed little tendency to adapt and behaved largely as tension receptors thus responding both to gastric distension and contraction

2) The existence of a gastro-gastric adrenergic inhibitory reflex was demonstrated with both afferent and efferent links conveyed by the splanchnic nerves and with the reflex coupling at the spinal level Also here the receptors particularly studied in the antral region behaved as slowly adapting tension receptors The efferent adrenergic link of the reflex acts essentially by inhibiting the excitatory cholinergic neurons in the gastric wall

3) These two distinctly separate suppressive gastro-gastric reflexes seem to be activated in close parallel in many situations and are jointly responsible for adjusting the gastric reservoir to its contents The vago-vagal gastro-gastric relaxatory reflex is here particularly important because of its pronounced relaxing effect that acts directly on the smooth muscle cells The considerable impairment of gastric reservoir capacity after vagotomy observed also in man supports this conclusion The gastro-gastric adrenergic inhibitory reflex complements however in an important way the vago-vagal reflex relaxation by modulating the activity of the cholinergic intramural excitatory neurons which during digestion elicit more or less rhythmic gastric contractions and propulsive movements Thus the two reflex mechanisms are likely to influence also gastric emptying by their effects on the aboral gastric pressure gradient

4) A pronounced gastric relaxation precedes vomiting in cats induced either by the centrally acting emetic drug apomorphine or by stimulation of heart receptor afferents In conscious dogs a similar gastric relaxation was observed in association with apomorphine induced nausea and vomiting This gastric relaxation was also mediated by the vagal

non-adrenergic relaxatory fibres, making the corpus-fundus part ready to receive the gastro-intestinal contents which are then expelled during vomiting The adrenergic inhibitory fibres are apparently of minor importance in these circumstances

ACKNOWLEDGEMENTS

To Professor Björn Folkow I wish to express my sincere gratitude for his *support and invaluable help throughout this study*

I am deeply grateful to docent Gunnar Jansson for the eminent way in which he introduced me into the field of gastrointestinal physiology and for his never-failing interest support and help during this work

I am also very grateful to docent Jan Martinson for invaluable help and many stimulating discussions His fundamental discoveries form an important basis for the present work

For stimulating and pleasant collaboration I wish to thank dr Peter Thorén

For skilful technical assistance I am indebted to Mrs Lisbeth Lindgren Mrs Dagny Johansson Mrs Eva Bengtsson Miss Pia Larsson and Mrs Eva-Carl Einarsson For typing the final manuscript I thank Mrs Kerstin Lundgren and Mrs Sylvia Palmquist

The valuable help of civ ing Lars Stage the photographer Tomas Palm and the animal keeper Gösta Magnusson is gratefully acknowledged

This research has been sponsored by grants from the Swedish Medical Research Council (B73-14X-16-09B and B72-61P-3607-01) from the Faculty of Medicine University of Göteborg and from Svenska Sällskapet för Medicinsk Forskning

REFERENCES

ABRAHAMSSON H Vago-vagal gastro-gastric relaxation Rendic R Gastroenterol 1971 3 114-115

ABRAHAMSSON H Reflex suppression of gastric motility elicited from antral mechanoreceptors Acta physiol scand 1973 87 40A-41A

ABRAHAMSSON, H and G JANSSON Elicitation of reflex vagal relaxation of the stomach from pharynx and esophagus in the cat Acta physiol scand 1969 77 172-178

ABRAHAMSSON H G JANSSON and J MARTINSON Vagal relaxation of the stomach induced by apomorphine Rendic R Gastroenterol 1971 3 114

ABRAHAMSSON H and P THORÉN Reflex relaxation of the stomach elicited from heart receptors Acta physiol scand 1970 80 8A-9A

ABRAHAMSSON, H and P THORÉN Reflex relaxation of the stomach elicited from receptors located in the heart An analysis of the receptors and afferents involved Acta physiol scand 1972 84 197-207

AGOSTONI E J E CHINNOCK M DE BURGH DALY and J G MURRAY Functional and histological studies on the vagus and its branches to the heart lungs and abdominal viscera in the cat J Physiol (Lond) 1957 135 182-205

ALVAREZ W C An introduction to gastroenterology Hoeber New York 1948

ARIMORI M C F CODE J F SCHLEGEL and R STURM Electrical activity of the canine esophagus and gastroesophageal sphincter Amer J dig Dis 1970 15 191-208

AUNE S Intragastric pressure after vagotomy in man Scand J Gastroent 1969 4 447-452

BARCLAY A E The digestive tract A radiological study of its anatomy physiology and pathology Cambridge University Press London 1936

BARRON L E and G M CURTIS The late effects of bilateral resection of the splanchnic nerves on the human gastric motor mechanism Amer J Physiol 1937 120 356-360

BAUMGARTEN H G Über die Verteilung von Catecholaminen im Darm des Menschen Z Zellforsch Mikroskop Anat 1967 83 133-146

BAYLISS W M and E H STARLING The movements and innervation of the small intestine J Physiol (Lond) 1899 24 99-143

BEANI L C BIANCHI and A CREMA Vagal non-adrenergic inhibition of guinea-pig stomach J Physiol (Lond) 1971 217 259-279

BEDI B S K A KELLY and K E HOLLEY Pathways of propagation of the canine gastric pacesetter potential Gastroenterology 1972 63 288-296

BOZLER, E Conduction automaticity and tonus of visceral muscles Experientia 1948 4 213-218

BURNSTOCK, G Purinergic nerves Pharmacol Rev 1972 24 509-581

BURNSTOCK G G CAMPBELL D G SATCHELL and A SMYTHE, Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut Brit J Pharmacol 1970 40 668-688

CAMPBELL G , The inhibitory nerve fibres in the vagal supply to the guinea-pig stomach J Physiol (Lond) 1966 185 600-612

CANNON, W B The movements of the stomach studied by means of the röntgen rays Amer J Physiol 1898 1 359-382

CANNON W B The mechanical factors of digestion Edward Arnold, London 1911

CANNON W B and C W LIEB The receptive relaxation of the stomach Amer J Physiol 1911 29 267-273

CARTER D C H N WHITEFIELD and I B McLEOD, The effect of vagotomy on gastric adaptation Gut 1972 13 874-879

CELANDER O Are there any centrally controlled sympathetic inhibitory fibres to the musculature of the intestine? Acta physiol scand 1959 47 299-309

CODE C F and H C CARLSON Motor activity of the stomach Handbook of Physiology Sect 6 Vol IV Amer Physiol Soc Washington 1968 1903-1916

DALY M and D H L EVANS Functional and histological changes in the vagus nerve of the cat after degenerative section at various levels J Physiol (Lond) 1953 120 579-595

DANIEL E E and J IRWIN Electrical activity of gastric musculature Handbook of Physiology Sect 6 Vol IV Amer Physiol Soc Washington 1968 1969-1984

DAVENPORT H W Physiology of the digestive tract Year Book Medical Publ Chicago 1971

DAVISON J S Response of single vagal afferent fibres to mechanical and chemical stimulation of the gastric and duodenal mucosa in cats Quart J exp Physiol 1972 57 405-416

EDVARDSEN P Nervous control of urinary bladder in cats I The collecting phase Acta physiol scand 1968 72 157-171

EDWARDS D A W and E N ROWLANDS Physiology of the gastroduodenal junction Handbook of Physiology Sect 6 Vol IV Amer Physiol Soc Washington 1968 1985-2000

FOLEY J O The functional types of nerve fibers and their numbers in the great splanchnic nerve Anat Rec 1948 100 766-767

GABELLA, G Synapses in the rat stomach and small intestine Experientia 1970 26 619-620

GABELLA G Synapses of adrenergic fibres Experientia 1971 27 280–281
GIANTURCO, G Some mechanical factors of gastric physiology Study I The empty stomach and its various ways of filling The pressure exerted by the gastric walls on the gastric contents The physical changes occurring to the food stuff during digestion Amer J Roentgenol 1934 31 735–744
GOLENHOFEN K D v LOH and K MILENOV Electrophysiologische Untersuchungen zur Spontanaktivität isolierter Muskelpräparate aus verschiedenen Abschnitten des Meerschweinchen-Magens Pflüg Arch ges Physiol 1970 315 336–356
GREEFF K H KASPERAT and W OSSWALD Paradoxe Wirkungen der elektrischen Vagusreizung am isolierten Magen- und Herzvorhofpräparat des Meerschweinchens sowie deren Beeinflussung durch Ganglienblocker Sympathicolytica Reserpin und Cocain Naunyn-Schmiedebergs Arch Pharmakol Exp Pathol 1962 243 528–545
GREY E G Observations on the postural activity of the stomach Amer J Physiol 1918 45 272-285
GROAT W C and P M LALLEY Reflex firing in the lumbar sympathetic outflow to activation of vesical afferent fibres J Physiol (Lond) 1972 226 289–309
GROAT W C and R W RYALL Reflexes to sacral parasympathetic neurones concerned with micturition in the cat J Physiol (Lond) 1969 200 87–108
HARDING R and B F LEEK Gastro-duodenal receptor responses to chemical and mechanical stimuli investigated by a single fibre technique J Physiol (Lond) 1972 a 222 139P-140P
HARDING R and B F LEEK Rapidly adapting mechanoreceptors in the reticulo-rumen which also respond to chemicals J Physiol (Lond) 1972 b 223 32P-33P
HESSE O Zur Kenntnis des Brechaktes Nach Röntgenversuchen an Hunden Pflügers Arch ges Physiol 1913 152 1–22
IGGO A Tension receptors in the stomach and the urinary bladder J Physiol (Lond) 1955 128 593–607
IGGO A Gastro-intestinal tension receptors with unmyelinated afferent fibres in the vagus of the cat Quart J exp Physiol 1957 a 42 130–143
IGGO A Gastric mucosal chemoreceptors with vagal afferent fibres in the cat Quart J exp Physiol 1957 b 42 398–409
JACOBOWITZ D Histochemical studies of the autonomic innervation of the gut J Pharmacol exp Ther 1965 149 358–364
JANSSON G Vago-vagal reflex relaxation of the stomach Acta physiol scand 1968 73 42A
JANSSON G Vago-vagal reflex relaxation of the stomach in the cat Acta physiol scand 1969 a 75 245–252

JANSSON G Extrinsic nervous control of gastric motility An experimental study in the cat Acta physiol scand 1969 b Suppl 326
JANSSON G and J MARTINSON Some quantitative considerations on vagally induced relaxation of the gastric smooth muscle in the cat Acta physiol scand 1965 63 351-357
JANSSON G and J MARTINSON Studies on the ganglionic site of action of sympathetic outflow to the stomach Acta physiol scand 1966 68 184-192
JOHANSSON B O JONSSON and B LJUNG Supraspinal control of the intestino-intestinal inhibitory reflex Acta physiol scand 1965 63 442-449
JOHANSSON B O JONSSON and B LJUNG, Tonic supraspinal mechanisms influencing the intestino-intestinal inhibitory reflex Acta physiol scand 1968 72 200-204
JOHANSSON B and J B LANGSTON, Reflex influence of mesenteric afferents on renal intestinal and muscle blood flow and on intestinal motility Acta physiol scand 1964 61 400-412
KELLING G Untersuchungen über die Spannungszustände der Bauchwand der Magen- und der Darmwand Z Biol 1903 44 161-267
KELLY K A and C F CODE, Canine gastric pacemaker Amer J Physiol 1971 220 112-118
KEWENTER J The vagal control of jejunal and ileal motility and blood flow Acta physiol scand 1965 Suppl 251
KÖSTER N and P MADSEN The intragastric pressure before and immediately after truncal vagotomy Scand J Gastroent 1970 5 381-383
LANGLEY J N On inhibitory fibres in the vagus to the end of the oesophagus and stomach J Physiol (Lond) 1898 23 407-414
LEEK B F Reticulo-ruminal mechanoreceptors in sheep J Physi (Lond) 1969 202 585-609
LEEK B F Abdominal visceral receptors Handbook of sensory ph logy Vol III Part I Enteroceptors Editor E Neil Spr Verlag Berlin 1971 116-160
LEEK B F The innervation of sheep forestomach papillae combined chemoreceptor and rapidly adapting responses are obtainable J Physiol (Lond) 1972
LIND J F H L DUTHIE J F SCHLEGEL and C F C ty of the gastric fundus Amer J Physiol 1961 201
LIPKIN M T ALMY and B M BELL Pressure-volume of the human colon J Clin Invest 1962 41 18
LUMSDEN K and W S HOLDEN The act of vom Gut 1969 10 173-179
MARTINSON J The effect of graded stimulation of nerve fibres on gastric motility Acta physiol scand

MARTINSON J Vagal relaxation of the stomach Experimental re-investigation of the concept of the transmission mechanism Acta physiol scand 1965 64 453–462

MARTINSON J and A MUREN Excitatory and inhibitory effects of vagus stimulation on gastric motility in the cat Acta physiol scand 1963 57 309-316

McSWINEY B A Innervation of the stomach Physiol Rev 1931 11 478-514

McSWINEY B A and W J WADGE, Effects of variations in intensity and frequency on the contractions of the stomach obtained by stimulation of the vagus nerve J Physiol (Lond) 1928 65 350-356

MEI N Mécanorécepteurs vagaux digestifs chez le chat Exp Brain Res 1970 11 502-514

MEI N J SALDUCCI H MONGES and F FARNARIER Afferent vagal impulses from the lower oesophageal sphincter of the cat Rendic R Gastroenterol 1972 4 65–68

MOBERG S G CARLBERGER F BARANY and G LUNDH Gastric emptying in peptic ulcer patients before and after partial gastrectomy and selective proximal vagotomy Rendic R Gastroenterol 1972 4 1-7

MORRISON J F B Mechanoreceptors in the region of the mesentery with A-delta and C-fibres in the splanchnic nerves of cats J Physiol (Lond) 1972 226 100P-101P

NAKAZATO Y and A OHGA Intramedullary pathways of the vago-vagal reflexes with special reference to those evoked by stimulation of the abdominal vagus Jap J Physiol 1971 21 175–188

NELSEN T S E H EIGENBRODT L A KEOSHIAN C BUNKER and L JOHNSON Alterations in muscular and electrical activity of the stomach following vagotomy Arch Surg 1967 94 821-835

NELSEN T S and S KOHATSU The stomach as a pump Rendic R Gastroenterol 1971 3 65–71

NIEMEGEERS C J E The apomorphine antagonism test in dogs Pharmacology 1971 6 353-364

NORBERG K A Adrenergic innervation of the intestinal wall studied by microscopy Int J Neuropharmacol 1964 3 379-382

ÖBERG, B and P THORÉN Increased activity in left ventricular receptors during hemorrhage or occlusion of caval veins in the cat A possible cause of the vaso-vagal reaction Acta physiol scand 1972 85 164-173

OHGA A Y NAKAZATO and K SAITO An analysis of the vago vagal reflex relaxation of the stomach J Physiol Soc Jap 1969 31 92-93

OHGA, A Y NAKAZATO and K SAITO, Considerations of the efferent nervous mechanism of the vago-vagal reflex relaxation of the stomach in the dog Jap J Pharmacol 1970 20 116-130
OPENCHOWSKI T , Ueber die nervösen Vorrichtungen des Magens Zbl Physiol 1889 3 1-10
PAINTAL A S , A study of gastric stretch receptors Their role in the peripheral mechanism of satiation of hunger and thirst J Physiol (Lond) 1954 126 255-270
PATON W D M and J R VANE An analysis of the responses of the isolated stomach to electrical stimulation and to drugs J Physiol (Lond) 1963 165 10-46
PATON W D M and E S VIZI The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig ileum longitudinal muscle strip Brit J Pharmacol 1969 35 10-28
RAMSBOTTOM N and J N HUNT Studies of the effect of metoclopramide and apomorphine on gastric emptying and secretion in man Gut 1970 11 989-993
SHARMA K N , H L JACOBS V GOPAL and S DUA-SHARMA Vago-sympathetic modulation of gastric mechanoreceptors Effect of distention and nutritional state J Neural Transmission 1972 33 113-154
SMITH C C and K R BRIZZEE Cineradiographic analysis of vomiting in the cat I Lower esophagus stomach and small intestine Gastroenterology 1961 40 654-664
STADAAS J and S AUNE Intragastric pressure/volume relationship before and after vagotomy Acta chir scand 1970 136 611-615
THOMAS J E Mechanics and regulation of gastric emptying Physiol Rev 1957 37 453-474
THOMAS J E and M V BALDWIN Pathways and mechanisms of regulation of gastric motility Handbook of Physiology Sect 6 Vol IV Amer Physiol Soc Washington 1968 1937-1968
-ORÉN P Left ventricular receptors activated by severe asphyxi and by coronary artery occlusion Acta physiol scand 1972 455-463
TITCHEN D A Reflex stimulation and inhibition of reticulu tractions in the ruminant stomach J Physiol (Lond) 1 1-21
WANG S C and H L BORISON A new concept of the central emetic mechanism Recent studies on the si of apomorphine copper sulfate and cardiac glycosides enterology 1952 22 1-12
WEISBRODT N W and J CHRISTENSEN Electrical acti cat duodenum in fasting and vomiting 1004-1010
WOLF S The relation of gastric function to nausea in Invest 1943 22 877-882